Microbial communities at a gasworks site: A marker gene- and cultivation-based approach to explore anaerobic aromatic compound degradation

Dissertation

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I ABBREVIATIONS

16S rRNA	Gene encoding for bacterial small ribosomal subunit
4-MBCR	4-Methylbenzoyl-CoA reductase (class I)
ACDB	Aromatic compound-degrading bacteria
bamA	Gene for 6-oxocylcohex-1-ene-1-carbonyl-CoA hydrolase
BCR	Benzoyl-CoA reductase (class I)
BTEX	Benzene, Toluene, Ethylbenzene, *p*-, *m*-, *o*-Xylene
CoA	Coenzyme A
DGGE	Denaturing gradient gel electrophoresis
DNAPL	Dense non-aqueous phase liquids
ENA	Enhanced natural attenuation
high-ox ACDB	High-oxygen-dependent aromatic compound-degrading bacteria
LNAPL	Light non-aqueous phase liquids
low-ox ACDB	Low-oxygen-dependent aromatic compound-degrading bacteria
MNA	Monitored natural attenuation
NA	Natural attenuation
no-ox ACDB	Oxygen-independent aromatic compound-degrading bacteria
PCR	Polymerase chain reaction
SIP	Stable isotope probing
T-RFLP	Terminal restriction fragment length polymorphism
US-EPA	United States Environmental Protection Agency

II SUMMARY

Environmental pollutants such as BTEX (benzene, toluene, ethylbenzene, *o*-, *m*-, *p*-xylene) and other aromatic compounds are frequently detected at gasworks sites. The aquifer of these sites are often oxygen-depleted and microorganisms are expected to drive the biodegradation processes under anaerobic conditions. In this study, anaerobic biodegradation processes were analyzed at a former Thuringia gasworks (i) *in situ* and (ii) by using nitrate-reducing enrichment cultures. The diversity and functioning of aromatic compound degrading bacteria (ACDB) was investigated by sequencing the marker genes 16S rRNA (universal marker for bacteria) and *bamA* (functional marker for anaerobic ACDB). With this approach, it was found that facultative anaerobic ACDB (mainly Betaproteobacteria) were well-established in the aquifer of the gasworks site. It was further found that facultative anaerobic bacteria co-occurred with obligate anaerobic sulfate- and/or CO_2 reducing ACDB (Deltaproteobacteria and Clostridia) in a high contaminated area of the plume, indicating that biodegradation processes are limited in this area by an insufficient supply of oxygen or nitrate. Several facultative anaerobic ACDB were investigated in more detail: *Azoarcus* spp. were key players in nitrate-dependent degradation of toluene, ethylbenzene and various other monoaromatic compounds. Bacteria related to the chemolithoautotrophic organism *Sulfuritalea hydrogenivorans* sk43H were high abundant and seem to have a so far overlooked role as denitrifying ACDB. This was deduced from the reanalysis of the published genome of *S. hydrogenivorans* sk43H and the finding that the organism utilizes simple aromatic carboxylate with nitrate. Bacteria related to *Sulfuritalea*, Comamonadacea and *Georgfuchsia* were enriched with *p*-alkylated aromatic carboxylates or *p*-xylene under nitrate-reducing conditions. This underlined the potential role of *Sulfuritalea* as an ACDB and was the first report about *Georgfuchsia* involved in *p*-xylene degradation. Noteworthy, evidences were found that *Azoarcus* spp. excrete high amounts of nitrite, which may drive the specialization of nitrite-sensitive ACDB for substrates that are not utilized by *Azoarcus* (i.e. *p*-alkylated aromatic compounds). This was the first systematic analysis of 16S rRNA and *bamA* sequences in environmental samples and enrichment cultures. Results from both marker genes were largely consistent and an implementation of 16S rRNA- and *bamA*-sequencing as routine diagnostic approach to assess redox processes, which are relevant for bioremediation, is suggested.

III ZUSAMMENFASSUNG

BTEX (Benzol, Toluol, Ethylbenzol, *o*-, *m*-, *p*-Xylol) und andere aromatische Umweltschadstoffe werden häufige an Gaswerksstandorten detektiert. Das Grundwasser dieser Standorte ist zumeist anoxisch und anaerobe Mikroorganismen sind mutmaßlich am biologischen Schadstoffabbau beteiligt. In dieser Arbeit wurden mikrobielle Abbauprozesse im Grundwasser eines ehemaligen Gaswerksstandortes in Thüringen (i) *in situ* und (ii) mit Hilfe nitratreduzierender Anreicherungskulturen untersucht. Die Diversität und Funktion aromantenabbauender Bakterien (ACDB) wurde mittels 16S rRNA (universeller Marker für Bakterien) und *bamA* (funktioneller Marker für anaerobe ACDB) Markergenanalysen untersucht. Es zeigte sich, dass fakultativ anaerobe ACDB (hauptsächlich Betaproteobakterien) am Gaswerksstandort etabliert sind. In einem hoch belasteten Bereich der Schadstofffahne waren zusätzlich Sulfat- und/oder CO_2 reduzierenden ACDB (Deltaproteobakterien und Clostridien) vorhanden. Diese deutet auf eine verminderte Aktivität der Abbauprozesse durch mangelnde Verfügbarkeit von Sauerstoff oder Nitrat. Die Rolle verschiedener fakultativ-anaerober ACDB wurde genauer untersucht: *Azoarcus* spp. waren Schlüsselorganismen beim nitratabhängigen Abbau von Toluol, Ethylbenzol und anderen monoaromatischen Verbindungen. Organismen mit Ähnlichkeit zu *Sulfuritalea hydrogenivorans* sk43H, einem chemolithoautotrophen Bakterium, waren hoch abundant. Sie scheinen eine bislang übersehene Rolle beim nitratabhängigen Abbau von Aromaten zu haben, was sich aus der Analyse des Genoms von *S. hydrogenivorans* sk43H und dessen Fähigkeit zum Umsatz von carboxylierten aromatischen Verbindungen mit Nitrat ergab. Bakterien mit Ähnlichkeit zu *Sulfuritalea*, Comamonadacea und *Georgfuchsia* wurden mit *p*-alkylierten aromatischen Carboxylaten oder *p*-Xylol angereichert. Dies unterstützt die potentielle Rolle von *Sulfuritalea* beim anaeroben Aromatenabbau und war der erste Hinweis auf eine Beteiligung von *Georgfuchsia* am Umsatz von *p*-Xylol. *Azoarcus* spp. scheiden große Mengen Nitrit aus, was eine Spezialisierung nitritsensitiver ACDB für Substrate begünstigen könnte, die nicht von *Azoarcus* abgebaut werden. Dies war die erste systematische Begutachtung biologischer Abbauprozessen mittels 16S rRNA and *bamA* Sequenzierung unter Verwendung von Umweltproben und Anreicherungskulturen. Die Resultate beider Markergene waren weitestgehend Deckungsleich. Eine Implementierung von 16S rRNA und *bamA* Markergenanalysen zur Routinediagnostik von Redoxprozessen, welche für die Bioremediation von Relevanz sind, wird empfohlen.

1 INTRODUCTION

1.1 AROMATIC COMPOUNDS IN NATURE AND AT GASWORKS SITES

Aromatic compounds are ideal building blocks of living material since they are exceptionally stable due to the high resonance energy levels of the circulating π electrons in the ring structure (Fuchs *et al*., 2011). Aromatic compounds are the main components of structural lignin, which is the second most abundant polymer on earth (Boerjan *et al*., 2003). They also play an important role in the secondary metabolism of plants, where they function as hormones, cofactors, defense compounds or attractants (Widhalm and Dudareva, 2015). Due to the stability of the aromatic ring system, these compounds tend to accumulate in the environment and end up as a major constituent in crude oil or coal. Aromatic compounds present in fossil fuels are industrially processed and used for combustion, as solvent or the synthesis of e.g. plastics, pesticides or pharmaceuticals. When released into the environment, they can be of major concern for human health (Schwarzenbach *et al*., 2010). A release of toxic aromatic compounds often occurs at former gasworks sites: more than 1,000 of such sites are recognized in Germany and the majority of them are unsecured (Mansfeldt, 2003). Many gasworks have an over 100 year old history of gas production, which was used for cooking and lightening, but most of them were abandoned after the replacement of coal gas by natural gas. The main side product of gas production was coal tar, which was often inadequately stored onsite in concrete pits from where toxic pollutants can leak into the groundwater. The composition of coal tar is diverse and comprises over 10,000 different compounds with up to 97% of the carbon belonging to aromatics (Zander *et al*., 1995). High molecular weight polycyclic aromatic hydrocarbons (PAH) have a low mobility in aquifers and are sinking as dense non-aqueous phase liquids (DNAPL) through the groundwater layers to the bottom of the aquifer. The main pollutants detected in the groundwater of gasworks sites are monocyclic BTEX compounds (Benzene, Toluene, Ethylbenzene, *p*-, *m*-, *o*-xylene), which float as light non-aqueous phase liquids (LNAPL) on top of the groundwater table (Leusch and Bartkow, 2010). Due to the small size of BTEX, they have a relatively good solubility in water and can form extensive contamination plumes. Bacteria control the extent of these plumes by biodegradation and the functioning of responsible metabolic pathways and the involved microorganisms are major fields of research.

1.2 PATHWAYS INVOLVED IN AROMATIC COMPOUND DEGRADATION

Bacterial degradation pathways for BTEX and other monocyclic aromatic compounds were reviewed by e.g. Harwood *et al*., 1996 and 1998; Vaillancourt *et al*., 2006; Carmona *et al*., 2009; Pérez-Pantoja *et al*., 2016; Weelink *et al*., 2010, Fuchs *et al*., 2011, Díaz *et al*., 2013 and Boll *et al*., 2014. Pathways involved in the degradation of selected model compounds and those relevant for the work presented here are depicted in Fig. 1A-D. The degradation of aromatic compounds can be divided in three major pathways: (1) Peripheral “activation” pathways comprise reactions that remove substituents attached to the ring and channel a large variety of aromatic compounds to few “activated” central intermediates. (2) Upper pathways facilitate the dearomatization and cleavage of the aromatic ring-systems. (3) In lower pathways, the products formed after ring-cleavage are further converted to intermediary metabolites such as acetyl-CoA, which is then used for microbial growth. The biodegradation of aromatic compounds occurs under aerobic, microaerobic and anaerobic conditions.

Aerobic pathways

Under aerobic conditions (Fig. 1A), degradation is initiated in peripheral pathways by oxygenases (oxidoreductases). These enzymes introduce destabilizing, electron-rich hydroxyl groups to the aromatic ring system, which leads to the formation of “activated” central intermediates such as catechol or protocatechuate. Dearomatization and ring-cleavage is catalyzed by dioxygenases (mostly Fe-dependent), which incorporate two oxygen atoms and are part of the upper pathway. After *ortho*-cleavage, the central intermediates are converted via β-ketoadipate to acetyl-CoA and succinate using the well-studied β-ketoadipate pathway (Harwood *et al*., 1996). Oxygen is also the terminal electron acceptor for respiration. At high concentrations of organic electron donors in contamination plumes, O_2 is rapidly consumed and microaerobic or anaerobic conditions prevail in polluted aquifers (Christensen *et al*., 1994).

Aerobic/Anaerobic hybrid pathways

Under microaerobic conditions, bacteria use the residual amounts of oxygen in hybrid pathways, which have aerobic and anaerobic characteristics. The peripheral “activation” in hybrid pathways is oxygen-independent and proceeds via arylcarboxyl-coenzyme A esters as central intermediates, which are also formed in anaerobic pathways. To overcome the high stabilizing resonance energy of aromatic rings, a single oxygen atom is incorporated. In case of the benzoyl-CoA dependent *box* pathway (Fig. 1B), the incorporation is catalyzed by the di-iron benzoyl-CoA monooxygenase (*boxAB*), which acts as an epoxidase (Rather

et al., 2010). The ring is cleaved by a benzoyl-CoA dihydrodiol lyase (*boxC*) and further converted to β-ketoadipyl-CoA, where the hybrid pathway converges with the β-ketoadipate pathway of aerobic aromatic compound degradation. The lower pathways do not require oxygen.

Anaerobic pathways

In the complete absence of oxygen, anaerobic degradation of aromatic compounds is also feasible, but occurs with lower rates. The anaerobic activation is initiated in peripheral pathways by enzymatic reactions that do not rely on oxygenases and which fundamentally differ to those involved under aerobic conditions (Fig. 1C). The underlying principles were reviewed by Boll *et al*., 2014: For toluene, the methyl side chain is added to fumarate by the glycyl radical-forming enzyme benzylsuccinate synthase (*bssABC*). For ethylbenzene, the ethyl side chain is oxidized under nitrate-reducing conditions by the molybdenum cofactor-containing ethylbenzene dehydrogenase (*ebdABCD*). The mechanistically very difficult conversion of benzene to benzoate is probably catalyzed by a CO_2-incorporating carboxylase. The degradation of *p*-cresol and phenol is initiated either by a flavocytochrome c hydroxylase that oxidizes the methyl side chain of *p*-cresol or by an ATP-dependent phenylphosphate carboxylase that introduces a carboxyl group to phenol. As part of the initial peripheral pathways, aromatic compounds are anaerobically activated by the formation of CoA thioesters. This activation is catalyzed by coenzyme A ligases, which introduce an electron-withdrawing carboxy-thioester group that facilitates subsequent ring-reduction of the central intermediate. In the upper pathway, the central intermediate benzoyl-CoA is reductively dearomatized by the ATP-dependent class I benzoyl-CoA reductase (BCR; *bcrABCD*/*bzdNOPQ*) in facultative anaerobic bacteria, or by an ATP-independent class II benzoyl-CoA reductase in obligate anaerobes. Ring reduction is catalyzed by a 6-oxocylcohex-1-ene-1-carbonyl-CoA hydrolase (*bamA*/*oah*), which is present in both, facultative and obligate anaerobic bacteria. In lower pathways, degradation proceeds via β-oxidation-like reactions and acetyl-CoA is formed, which is used for microbial growth. The anaerobic degradation of *p*-xylene proceeds via a similar pathway as for toluene, however, the *para*-positioned methyl group is retained throughout the entire pathway (Fig. 1D). The critical step for the degradation of *p*-xylene and other *p*-alkylated compounds is the reductive dearomatization of *p*-alkylated benzoyl-CoA intermediates. The dearomatization requires ring protonation in *para*-position to the carboxy thioester group, which is sterically hindered in conventional class I benzoyl-CoA reductases (BCR) (Möbitz and Boll, 2002) Therefore, the reduction of the central intermediate 4-methylbenzoyl-CoA requires a specific 4-methylbenzoyl-CoA reductase (4-MBCR; *mbrABCD*) (Lahme *et al*., 2012).

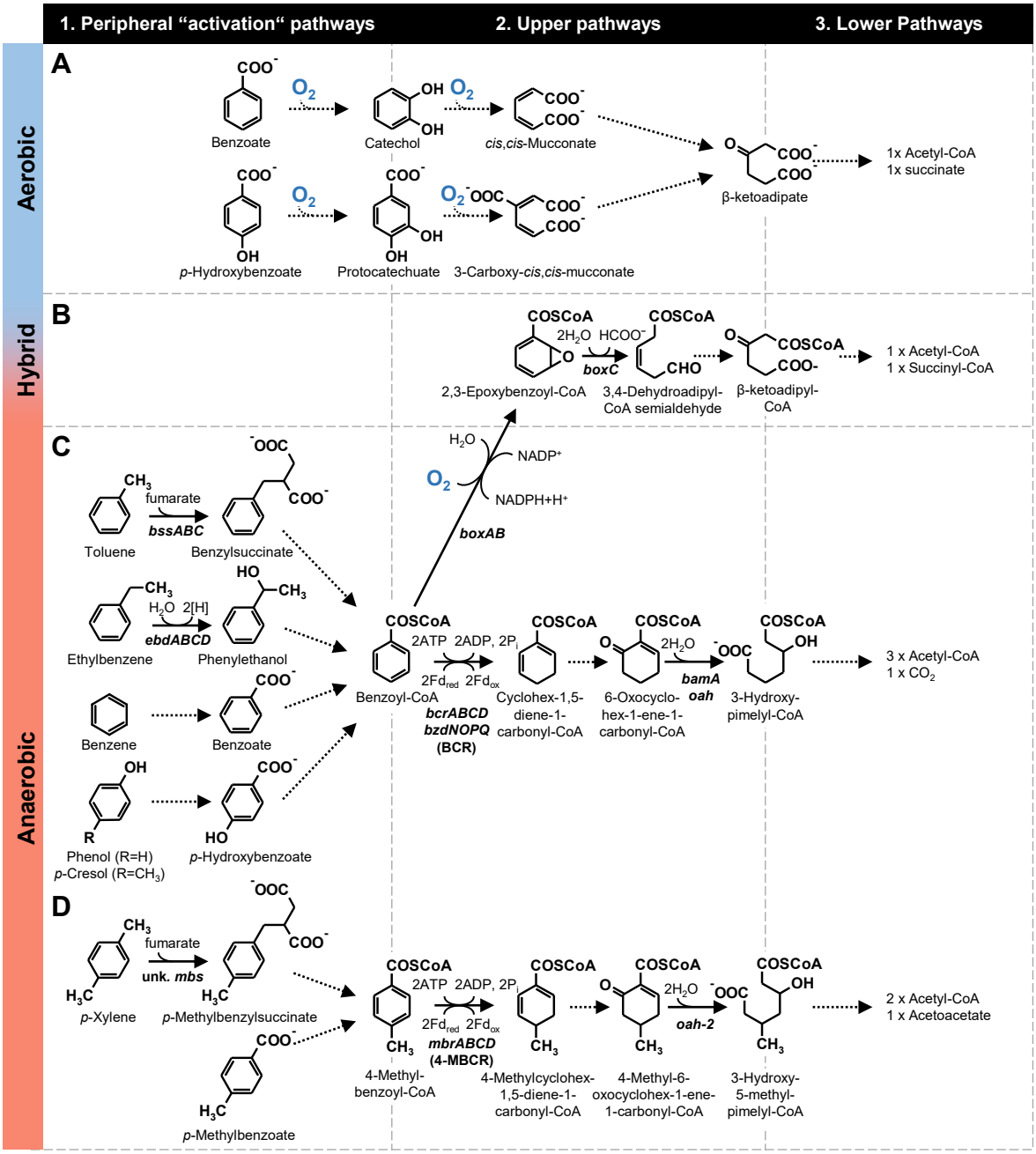

Figure 1: Bacterial pathways involved in aromatic compound degradation exemplified for selected model compounds. Aerobic and anaerobic degradation proceeds via (1) a peripheral "activation" pathway, (2) an upper dearomatization and ring-cleaving pathway, and (3) a lower pathway. **A**: Under aerobic conditions, aromatic compounds are hydroxylated by oxygenases and channeled to "activated" central intermediates such as catechol or protocatechuate. Dearomatization and ring-cleavage is catalyzed by dioxygenases. In case of *ortho*-cleavage, degradation proceeds via β-ketoadipate to acetyl-CoA and succinate using the β-ketoadipate pathway. **B**: The aerobic/anaerobic hybrid pathway uses "activated" arylcarboxyl-CoA esters such as benzoyl-CoA as central intermediates. For dearomatization, an epoxide-forming benzoyl-CoA monooxygenase complex is involved (*boxAB*). The ring is cleaved by a benzoyl-CoA dihydrodiol lyase (*boxC*) and the product is further converted via β-ketoadipyl-CoA to acetyl-CoA and succinyl-CoA. **C**: Under anaerobic conditions, oxygen-independent reactions initiate aromatic compound degradation: for toluene, the methyl side chain is added to fumarate by a benzylsuccinate synthase (*bssABC*). The ethyl side chain of ethylbenzene is oxidized by an ethylbenzene dehydrogenase (*ebdABCD*). Benzene is probably activated to benzoate by a carboxylase. The degradation of *p*-cresol and phenol requires a flavocytochrome c hydroxylase or an ATP-dependent phenylphosphate carboxylase, respectively. CoA ligases catalyze the anaerobic activation of aromatic compounds to the "activated" central intermediate benzoyl-CoA. Dearomatization is catalyzed either by an ATP-dependent class I benzoyl-CoA reductase (*Thauera*: *bcrABCD*; *Azoarcus*: *bzdNOPQ*) or by an ATP-independent class II benzoyl-CoA reductase (*Geobacter*: *bamBCDEFGHI*; not depicted). The ring is cleaved by a 6-oxocylcohex-1-ene-1-carbonyl-CoA hydrolase (Geobacter: *bamA; Thauera* and *Magnetospirillum*: *oah*). The metabolic end-product is acetyl-CoA, which is formed via β-oxidation-like reactions. **D**: Degradation of *p*-xylene is similar as for toluene. It involves an unknown (4-methylbenzyl)succinate synthase (unk. *mbs*), a specific 4-methylbenzoyl-CoA reductase (*mbrABCD*) and a second ring-cleaving hydrolase (*oah-2*). Adapted from Harwood *et al*., 1996 and 1998, Carmona *et al*., 2009, Fuchs *et al*.,2011, Lahme *et al*., 2012, Boll *et al*., 2014. Solid arrows: a single reaction is involved. Dashed arrows: different reactions may be involved and participating reactants are not shown.

1.3 AROMATIC COMPOUND-DEGRADING BACTERIA

Only microorganisms can fully mineralize aromatic compounds. Aromatic compound-degrading bacteria (ACDB) can be distinguished by their oxygen demand and were defined in this work as high-oxygen- (high-ox), low-oxygen- (low-ox) and oxygen-independent ACDB (no-ox).

High-ox ACDB

Members of the high-ox ACDB group do not degrade aromatic compounds with electron acceptors different from oxygen. High-ox ACDB mostly rely on oxygenase-dependent aerobic pathways. They may also encode aerobic/anaerobic hybrid pathways but never possess anaerobic pathways (Fig. 1A-D). The aerobic degradation of aromatic compounds by bacterial isolates is known for a long time (Söhngen, 1913). Using modern molecular techniques, it became apparent that aerobic degraders are phylogenetically diverse. Aerobic catabolic pathways frequently occur in the phyla Proteobacteria and Actinobacteria, but also in Bacteroidetes, Chloroflexi, Firmicutes, Acidobacteria and members of the Thermus/Deinococcus (Pérez-Pantoja *et al*., 2016; Vilchez-Vargas *et al*., 2010).

Low-ox ACDB

These bacteria may degrade aromatic compounds under microaerobic conditions or with alternative electron acceptors such as nitrate. For example, members of the genera *Dechloromonas* and *Rhodoferax* utilize aromatic compounds under nitrate- or iron-reducing conditions, but do not possess known anaerobic degradation pathways (Risso *et al*., 2009; Salinero *et al*., 2009). Anaerobic pathways are always absent in low-ox ACDB, but they often encode aerobic/anaerobic hybrid pathways. Hybrid pathways are considered as an adaptation to low or fluctuating oxygen concentrations. They occur in about 5% of all bacteria with a sequenced genome and are mostly found in Proteobacteria and Actinobacteria (Rather *et al*., 2010; Valderrama *et al*., 2012).

No-ox ACDB

Oxygen-independent bacteria always possess anaerobic pathways for aromatic compound degradation (Fig. 1C-D). The presence of aerobic and/or hybrid pathways is possible (Fig. 1A-B), as observed e.g. for the facultative anaerobic model organism "*Aromatoleum aromaticum*" EbN1 (Rabus *et al*., 2005). Based on the conditions at the gasworks site, the focus of this study was set on no-ox ACDB that utilize BTEX and other monoaromatic compounds in the absence of oxygen. Facultative and obligate anaerobic no-ox ACDB were summarized in several recent reviews (Widdel *et al*., 2010, Weelink *et al*., 2010; Kleinsteuber *et al*., 2012; Boll *et al*., 2014; Lueders, 2016). All recognized no-ox ACDB

belong to the phyla Proteobacteria (classes: Alpha-, Beta-, Deltaproteobacteria) or Firmicutes (class: Clostridia). Also a single Archeaon was described (Holmes *et al*., 2011). No-ox ACDB are distinguished by their utilized electron acceptors, which are e.g. nitrate, Fe(III), Sulfate or CO_2. Nitrate is often utilized by facultative anaerobic Betaproteobacteria, including well-studied members of the denitrifying genera *Azoarcus* and *Thauera* (recently reclassified from the familiy *Rhodocyclaceae* to *Zoogloeaceae*; Boden *et al*., 2017). The toluene- and ethylbenzene-degrading Betaproteobacterium *Georgfuchsia toluolica* (family: Sterolibacteriaceae) uses either iron(III), nitrate or manganese(IV) as electron acceptor (Weelink *et al*., 2009; Oosterkamp, 2013). Other nitrate-reducing no-ox ACDB belong to Alphaproteobacteria such as nitrate-reducing *Magnetospirillum* spp. (family: Rhodospirillaceae; Shinoda *et al*., 2005). Photoautothrophic benzoate utilization was shown in the Alphaproteobacterium *Rhodopseudomonas palustris* (Egland *et al*., 1997). No-ox ACDB that reduce Fe(III), sulfate or CO_2 are mostly obligate anaerobic bacteria. Obligate anaerobic Fe(III)-reducing bacteria belong to the genera *Geobacter* (family: Geobacteraceae; class: Deltaproteobacteria) or *Desulfitobacterium* (familiy: Peptococcaceae; class Clostridia; Kunapuli *et al*., 2010). No-ox ACDB that utilize BTEX compounds with sulfate were enriched and/or isolated from marine and terrestrial habitats. Marine sulfate-reducing ACDB are related to members of the deltaproteobacterial genera *Desulfosarcina*, *Desulfobacula* or *Desulfotignum* (family: Desulfobacteraeae) (Rabus *et al*., 1993; Harms *et al*., 1999; Ommedal and Torsvik, 2007). Terrestrial sulfate-reducing bacteria are related to the deltaproteobacterial genera *Desulforhabdus* (family: Syntrophobacteraceae) and *Desulfocapsa* (family: Desulfobulbaceae) as well as to the clostridial genera *Desulfotomaculum* and *Desulfosporosinus* (family: Peptococcaceae) (Beller *et al*., 1996; Meckenstock 1999; Liu *et al*., 2004; Morasch *et al*., 2005; Kleinsteuber *et al*., 2008; Berlendis *et al*., 2010; Abu Laban *et al*., 2015a and 2015b). Under fermenting conditions, no-ox ACDB include members of the genera *Pelotomaculum* (family Peptococcaceae, class: Clostridia) or *Syntrophus* (family: Syntrophaceae; class: Deltaproteobacteria), which co-occur in syntrophic interactions with methanogenic Archaea (Mountfort *et al*., 1984; Jackson *et al*., 1999; Ulrich and Edwards, 2003; Qiu *et al*., 2006; Sakai *et al*., 2009; Fowler *et al*., 2012). The number of organisms that are described to utilize aromatic compounds via oxygen-independent reactions is steadily increasing.

1.4 ASSESSMENT OF BIODEGRADATION PROCESSES

Bioremediation is based on the capability of indigenous microorganisms to remove or immobilize pollutants by biodegradation. Biodegradation processes are defined by the US-EPA as “natural attenuation” (NA) and bioremediation strategies involve the monitoring of

NA (MNA: monitored natural attenuation) or the stimulation of NA (ENA: enhanced natural attenuation). Also the “artificial” addition of microorganisms is possible (bioaugmentation). Bioremediation strategies are often based on oxygen-dependent biodegradation processes, but also anaerobic approaches can be applied, which are less disturbing to oxygen-sensitive microorganisms already established at anoxic sites (reviewed by e.g. Bamforth and Singleton, 2005; Farhadian *et al*., 2008; Perelo, 2010; Megharaj *et al*., 2011; Gieg *et al*., 2014; Koenig *et al*., 2014). The success of bioremediation strategies depends on the presence and functioning of degrading microorganisms and therefore, a prerequisite for the implementation of a suitable strategy includes the assessment of indigenous microorganisms (Majone *et al*., 2015). In the scope of the BMBF funding priority KORA, several microbiological methods were recommended that allow such an assessment (Michels *et al*., 2008). The underlying principles of these methods are summarized in the following: In classical culture-dependent approaches, microorganisms are enriched or isolated by incubating environmental samples in different growth media. These growth media are selective e.g. for organisms that degrade specific organic contaminants or thrive under different electron accepting conditions. Microorganisms are quantified by e.g. counting the number of colony forming units (CFU) on agar plates, by determining the most probable number (MPN) after sequential dilution series or by measuring substrate utilization patterns (Alef and Kleiner, 1989; Garland, 1996; Morasch *et al*., 2001; Tiehm *et al*., 2005; Figueras and Borrego, 2010). The BACTRAP® method allows *in-situ* cultivations; a ^{13}C-labled contaminant of interest is spotted on a carrier material and then incubated in the groundwater for several weeks. Microorganisms that degrade the labeled substrate enrich at the carrier surface and incorporate ^{13}C-labled carbon into their biomass, which can be detected by mass spectrometry (Stelzer *et al*., 2006). As a major drawback, culture-dependent methods are often time-consuming and fail to detect “unculturable” organisms. Culture-independent methods allow to analyze the *in situ* communities at contaminated sites without a preceding cultivation step. Microbial *in situ* communities can be examined under the microscope by staining them either unselectively with fluorophores such as DAPI or by using FISH probes that are specific for selected organisms (Kepner and Pratt, 1994; Amann *et al*., 1997). Biodegradation can be further assessed by the chemical analysis of metabolites that are characteristic for biotic degradation pathways or by determining isotopic fractionation patterns (Gieg *et al*., 2002; Griebler *et al*., 2004; Knöller *et al*., 2006; Reineke *et al*., 2007; Fischer *et al*., 2008; Musat *et al*., 2016). As an additional approach, the analysis of marker genes allows to detect microorganisms and pathways that are potentially involved in biodegradation. The 16S rRNA gene is a well-established universal marker to study bacterial or archaeal diversity (Guo *et al*., 2013; Henschel *et al*., 2015;

Fischer *et al*., 2016). Numerous functional marker genes were established to detect e.g. sulfur- and nitrogen-cycling, methane oxidation, or dioxygenase-dependent aerobic pathways for aromatic compound degradation (Iwai *et al*., 2011; Imhoff, 2016). To analyze anaerobic aromatic compound degradation, three different genes are targeted: (i) The benzylsuccinate synthase gene (*bssA*) is present in no-ox ACDB that degrade toluene or xylenes, (ii) the class I or class II benzoyl-CoA reductase (*bcrC*/*bzdN* and *bamB*) is present in facultative anaerobic or obligate anaerobic ACDB, respectively, and (iii) the ring-cleaving hydrolase (*bamA*/*oah*) is conserved in most no-ox ACDB (see p. 4, Fig. 1C; Winderl *et al*., 2007; Kuntze *et al*., 2011; Staats *et al*., 2011; von Netzer *et al*., 2016). The marker genes can be e.g. qualitatively analyzed by PCR amplification, fingerprinted by DGGE or T-RFLP, quantified by real-time PCR or sequenced by using clone libraries or next-generation sequencing (e.g. Iwamoto *et al*., 2000; Beller *et al*., 2002; Tischer *et al*., 2013; Fahrenfeld *et al*., 2014; Tan *et al*., 2015).

1.5 AIMS OF THE STUDY

The overall aim of the study was to contribute to a better understanding of biodegradation processes that occur at aromatic compound polluted sites. In the course of the BMWi funded ZIM project REMINVAS (grant number: KF2026007RH2), a novel bioremediation strategy was developed at the BTEX contaminated Thuringia gasworks, which included an approach to stimulate anaerobic biodegradation processes with nitrate. To evaluate the potential for the implementation of such a strategy, an experimental setup was designed to answer the following questions: Which microorganisms are present at the gasworks site and which of them are potentially involved in anaerobic aromatic compound degradation? Can BTEX-degrading microorganisms be stimulated with nitrate, and if yes, which organisms are involved? Are potential no-ox ACDB abundant that were not recognized as anaerobic aromatic compound degrading bacteria before? Which aromatic substrates are utilized by anaerobic key players? Which electron-accepting processes occur along a horizontal pollutant gradient? To answer these question, a combination of culture-dependent and marker gene-based culture-independent methods were applied. The 16S rRNA and *bamA* marker genes were analyzed for their diversity; the former is a universal marker gene aimed to detect the majority of bacteria, and the latter is a functional marker to detect no-ox ACDB (Fig. 1C). At the end of the thesis, the applicability of this approach as a possible blueprint to assess biodegradation processes is discussed.

2 MANUSCRIPTS

2.1 OVERVIEW

2.1.1 Manuscript I

Title: Microbial community of a gasworks aquifer and identification of nitrate-reducing *Azoarcus* and *Georgfuchsia* as key players in BTEX degradation

Authors: Sperfeld M, Rauschenbach C, Diekert G, Studenik S

Current state: Published in *Water Research* (2018), vol. 132, p. 146-157

Supplementary materials: see chapter V, p. i - xi

Summary: The main pollutants in the groundwater of the Thuringia gasworks site are BTEX compounds. We analyzed the microbial community in the groundwater along a pollutant gradient by 16S rRNA (universal marker for bacteria) and *bamA* (marker for anaerobic aromatic compound degradation) gene marker sequencing as well as by nitrate-reducing enrichment cultures. We found that up to 44% of the bacterial 16S rRNA sequences belonged to anaerobic aromatic compound-degrading bacteria (ACDB). A hotspot for sulfate-dependent ACDB was present in the highest contaminated groundwater well, whereas nitrate-reducing ACDB were equally distributed along the pollutant gradient. Key players for nitrate-dependent BTEX degradation were members of the genus *Azoarcus*, which were high abundant and readily enriched with toluene and ethylbenzene. Members of the genus *Georgfuchsia* were low abundant, but outcompeted *Azoarcus* with *p*-xylene as electron donor. This was the first report about a *Georgfuchsia*-affiliated organism involved in *p*-xylene degradation, a compound that is often reported to be particularly recalcitrant. The applied methods allowed a functional and thorough characterization of microbial biodegradation processes established in the aquifer of the Thuringia gasworks site.

Contribution: My own contribution to this manuscript covers about 70%. I was involved in the planning of the experimental setup, conducted all microbial cultivations and analyzed substrate utilization by HPLC, GC and colorimetric assays. I performed DNA extractions, prepared the majority of *bamA*-clone libraries, reanalyzed 16S rRNA Illumina data obtained from a private company and designed degenerated primers to detect a putative *p*-methylbenzoyl-CoA reductase gene cluster. I conducted an extensive literature search regarding aromatic compound-degrading bacteria, wrote the first version of the manuscript and participated in the revision process.

2.1.2 Manuscript II

Title: Anaerobic aromatic compound degradation in *Sulfuritalea hydrogenivorans* sk43H

Authors: Sperfeld M, Diekert G, Studenik S

Current state: Submitted to *FEMS Microbiology Ecology*, date of submission: 09/May/2018

Supplementary materials: see chapter V, p. xii - xvi

Summary: Bacteria related to *Sulfuritalea hydrogenivorans* sk43H were highly abundant at the Thuringia gasworks site, as previously shown by 16S rRNA deep sequencing (up to 10.7 % of sequences) and *bamA* clone library analysis (up to 23.3 % of clones). The *bamA* gene is a marker for anaerobic aromatic compound degradation and we hypothesized that *Sulfuritalea*-related organisms are involved in anaerobic aromatic compound degradation at this gasworks site. In general, this organism is recognized as sulfur-oxidizing bacterium that utilizes nitrate as electron acceptor. An involvement in anaerobic aromatic compound degradation was not anticipated. Therefore, we (i) reanalyzed environmental studies for the occurrence of *Sulfuritalea* spp., (ii) reanalyzed the published genome of *S. hydrogenivorans* sk43H for pathways possibly involved in aromatic compound degradation and (iii) tested the utilization of aromatic substrates by strain sk43H under nitrate-reducing conditions. We found that *Sulfuritalea* spp. frequently occur at aromatic compound contaminated sites and that *S. hydrogenivorans* sk43H utilizes simple aromatic carboxylates including benzoate, phenylacetate and *p*-hydroxylated analogues. These compounds are plant-derived or can be excreted by e.g. *Azoarcus* spp. during the utilization of industrially produced aromatics such as phenol or *p*-cresol. We presented a genetic and physiological basis for anaerobic aromatic compound degradation in *Sulfuritalea* sp., which will allow more refined interpretations about the role of related organisms at aromatic compound polluted sites in the future.

Contributions: My own contribution to this manuscript covers about 90%. I planned most of the experimental setup, conducted microbial cultivations, measured substrate utilization by GC and HPLC, did a literature search regarding *Sulfuritalea*-related organisms, and analyzed the published genome of *Sulfuritalea hydrogenivorans* sk43H for pathways putatively involved in aromatic compound degradation. I further wrote the first version of the manuscript and was involved in discussions.

2.1.3 Manuscript III

Title: Community dynamics in a nitrate-reducing microbial consortium cultivated with *p*-alkylated vs. non-*p*-alkylated aromatic compounds

Authors: Sperfeld M, Diekert G, Studenik S.

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Supplementary materials: see chapter V, p. xvii - xxi

Summary: The anaerobic degradation of *p*-alkylated aromatic compounds is considered as challenging due to mechanistic constraints that occur during aromatic ring reduction. Surprisingly, *p*-xylene was utilized with higher rates than *o*- and *m*-xylene in nitrate-reducing enrichment cultures from the Thuringia gasworks site. We hypothesized that a microbial community is present, which is well-adapted to the anaerobic degradation of *p*-alkylated aromatic compounds. Following this, we established enrichment culture pMB18, which utilized *p*-methylbenzoate and other *p*-alkylated carboxylic acids under nitrate-reducing conditions. Bacteria affiliated to *Sulfuritalea* and Comamonadaceae were most likely involved in the degradation of *p*-alkylated aromatic compounds. When *p*-alkylated compounds were replaced by non-*p*-alkylated analogues, the *Sulfuritalea*/Comamonadaceae community was suppressed and *Azoarcus*-affiliated bacteria became abundant. We found evidences that the *Sulfuritalea*/Comamonadaceae community is inhibited by *Azoarcus*-dependent excretions of nitrite. It can be speculated that *Azoarcus*-dependent nitrite-excretions drive the specialization of nitrite-sensitive ACDB for substrates that are not utilized by *Azoarcus*. A specialization for *p*-alkylated aromatic substrates would require e.g. a specific 4-methylbenzoyl-CoA reductase (4-MBCR) to dearomatize *p*-methylated aromatic ring system. In accordance, a putative 4-MBCR coding gene region was detected in culture pMB18 cultivated with *p*-methylbenzoate, but not in an *Azoarcus*-dominated culture grown with the non-*p*-alkylated compound benzoate.

Contributions: My own contribution to this manuscript covers about 80%. I planned most of the experiments, conducted all microbial cultivations, quantified substrate utilization by HPLC and GC, reanalyzed 16S rRNA sequencing data obtained from a private sequencing company and performed *in-silico* analyses of *bamA* sequences. I wrote the first version of the manuscript and was involved in discussions.

2.2 MANUSCRIPT I

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Microbial community of a gasworks aquifer and identification of nitrate-reducing *Azoarcus* and *Georgfuchsia* as key players in BTEX degradation

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ABSTRACT

We analyzed a coal tar polluted aquifer of a former gasworks site in Thuringia (Germany) for the presence and function of aromatic compound-degrading bacteria (ACDB) by 16S rRNA Illumina sequencing, *bamA* clone library sequencing and cultivation attempts. The relative abundance of ACDB was highest close to the source of contamination. Up to 44% of total 16S rRNA sequences were affiliated to ACDB including genera such as *Azoarcus, Georgfuchsia, Rhodoferax, Sulfuritalea* (all Betaproteobacteria) and *Pelotomaculum* (Firmicutes). Sequencing of *bamA*, a functional gene marker for the anaerobic benzoyl-CoA pathway, allowed further insights into electron-accepting processes in the aquifer: *bamA* sequences of mainly nitrate-reducing Betaproteobacteria were abundant in all groundwater samples, whereas an additional sulfate-reducing and/or fermenting microbial community (Deltaproteobacteria, Firmicutes) was restricted to a highly contaminated, sulfate-depleted groundwater sampling well. By conducting growth experiments with groundwater as inoculum and nitrate as electron acceptor, organisms related to *Azoarcus* spp. were identified as key players in the degradation of toluene and ethylbenzene. An organism highly related to *Georgfuchsia toluolica* G5G6 was enriched with *p*-xylene, a particularly recalcitrant compound. The anaerobic degradation of *p*-xylene requires a metabolic trait that was not described for members of the genus *Georgfuchsia* before. In line with this, we were able to identify a putative 4-methylbenzoyl-CoA reductase gene cluster in the respective enrichment culture, which is possibly involved in the anaerobic degradation of *p*-xylene.

1. Introduction

In Germany, at least 1000 former gasworks sites are known (Mansfeldt, 2003), which are potential sources of pollutants that pose a risk to human health. The main contaminations at these sites derive from inappropriately stored coal tar, which is a complex mixture of mostly aromatic compounds (Zander, 1995). Especially BTEX (benzene, toluene, ethylbenzene, and xylenes) are of concern, since these low molecular weight toxic compounds are mobile with the groundwater flow. In general, the biodegradation of BTEX can occur under oxic conditions, or at reduced rates in the absence of oxygen with alternative electron acceptors such as nitrate, sulfate, Fe(III) or CO_2 (for reviews see Weelink et al., 2010; Fuchs et al., 2011). Contaminated aquifers are usually oxygen depleted and therefore facultative and/or obligate anaerobic bacteria are assumed to dominate the aromatic compound-degrading communities. All of the so far described isolates of aromatic compound-degrading anaerobes belong to the Proteobacteria or Firmicutes (for reviews see Widdel et al., 2010; Kleinsteuber et al., 2012; Boll et al., 2014; Lueders, 2017). Within the Proteobacteria, *Magnetospirillum* spp. (Alphaproteobacteria), *Azoarcus* spp. and *Thauera* spp. (both Betaproteobacteria) degrade aromatic compounds under nitrate-reducing conditions (Evans et al., 1991; Fries et al., 1994; Anders et al., 1995; Rabus and Widdel, 1995; Shinoda et al., 2005). The use of Fe(III) as electron acceptor was mainly reported for *Geobacter* spp. (Deltaproteobacteria) (Lovley and Lonergan, 1990; Coates et al., 2001), but also for *Georgfuchsia* spp. (Betaproteobacteria), which additionally uses nitrate or Mn(IV) as electron acceptor (Weelink et al., 2009). Sulfate is common for Desulfobulbaceae and Desulfobacteraceae (both Deltaproteobacteria) (Bak and Widdel, 1986;

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Meckenstock, 1999) as well as for *Desulfotomaculum* spp. and *Desulfosporosinus* spp. (Clostridia, Firmicutes) (Cord-Ruwisch and Garcia, 1985; Sun et al., 2014a). Members of the genus *Pelotomaculum* (Clostridia, Firmicutes) were often found in syntrophic consortia, in which aromatic compound degradation was observed under sulfate- or nitrate-reducing or fermenting conditions (for review see Kleinsteuber et al., 2012).

An important field of research focuses on the bioremediation of aromatic compound-contaminated sites by e.g. stimulating microbial attenuation processes (for reviews see Bamforth and Singleton, 2005; Farhadian et al., 2008; Perelo, 2010; Megharaj et al., 2011). For the design and monitoring of bioremediation approaches, it is important to identify the microorganisms present at the site and to predict their possible function in pollutant degradation (Majone et al., 2015). State of the art sequencing technologies allow an affordable in-depth analysis of 16S rRNA gene diversity (e.g. Langille et al., 2013; Henschel et al., 2015; Tan et al., 2015) and classified 16S rRNA sequences can be screened for the presence of potential aromatic compound-degrading bacteria; however, the corresponding microorganisms do not necessarily possess the genes for biodegradation (Junca and Pieper, 2004). To specifically detect aromatic compound-degrading anaerobes, a number of primers have been established that target functional genes of pathways involved in aromatic compound degradation (for review see von Netzer et al., 2016). These genes are (i) *bssA*, (ii) *bcr*/*bzd* or *bamB*, and (iii) *bamA* (Beller et al., 2002; Hosoda et al., 2005; Song and Ward, 2005; Winderl et al., 2007; Kuntze et al., 2008; 2011; Löffler et al., 2011; Staats et al., 2011). The *bssA* gene encodes a benzylsuccinate synthase that oxidizes alkyl side chains of substituted aromatic compounds by the addition of fumarate, a mechanism applied for the activation of most BTEX. The genes *bcr*/*bzd* or *bamB* code for ATP-dependent or -independent arylcoenzyme A reductases that catalyze the dearomatization of the aromatic ring. Together with *bamA*, which encodes a ring-cleaving hydrolase, *bcr*/*bzd* and *bamB* target the central catabolic pathway for anaerobic degradation of aromatic compounds such as petrochemicals, humic acids, lignin monomers, and aromatic amino acids (Carmona et al., 2009). The conserved *bamA* gene finds increasing application as functional gene marker, since it is suitable for the simultaneous detection of facultative and obligate anaerobic aromatic compound degraders and can be frequently PCR-amplified from different habitats. Sequences of *bamA* were either retrieved from field sites (Kuntze et al., 2011; Staats et al., 2011; Porter and Young, 2013; Verde et al., 2013; Ruan et al., 2016) or from microbial cultures growing with different electron acceptors (Kuntze et al., 2008; Higashioka et al., 2011; Li et al., 2012; Sun et al., 2014b).

In this study, 16S rRNA and *bamA* sequencing were applied for the functional description of a microbial community of a coal-tar polluted aquifer. This approach allowed a rapid and easy identification of potential aromatic compound-degrading bacteria as well as the prediction of prevailing electron-accepting processes in the aquifer. Further, a cultivation-based approach was used to identify microorganisms involved in the nitrate-dependent bioremediation of BTEX. This is the first combined application of deep 16S rRNA sequencing, *bamA* profiling, and cultivation attempts to characterize a microbial community of a BTEX-contaminated site.

2. Material and methods

2.1. Sampling site

The sampling site was a former gasworks area located in Thuringia (Germany), which was not remediated so far. Coal tar derived pollutants are still leaking into the groundwater. The geology of the test field was analyzed by the JENA-GEOS®-Ingenieurbüro GmbH (Jena, Germany). The gasworks area is characterized by horizontal layers of an anthropogenic backfill at the top (2–3 m thickness) followed by alluvial loam (1 m thickness, Holocene), a layer of gravel (2–2.5 m thickness, Pleistocene) and sandstone sediment (start 5–6 m below ground, Rotliegend). The loam functions as aquiclude with low water permeability. In general, the loam hampers the infiltration of rainwater and pollutants from the anthropogenic backfill into deeper layers; however, it was partially removed below buildings located at the site. The layer of gravel represents the main water-bearing aquifer. The approximate groundwater flow rate is 10 cm per day. Groundwater was sampled from three wells (2 inch in diameter) originally designated as P2/98, RP 12/12 and P1/98 and renamed for convenience to A, B, and C, respectively. The wells are located downstream from the coal tar pit along a pollution gradient (Fig. 1). The wells have a depth of about six meter below ground with a 1 m screen section (5–6 m below ground; 1 mm pore size), which covers approximate half of the thickness of the aquifer. Groundwater samples were collected between 2012 and 2014 by JENA-GEOS®-Ingenieurbüro GmbH (Jena, Germany). Before sampling, groundwater was pumped for 10 min and discarded. Subsequently, the following parameters were determined on-site: oxygen, electrical conductivity, pH, redox potential and temperature. Further groundwater parameters and compound concentrations were determined by the Thüringer Umweltinstitut Henterich GmbH & Co. KG (Krauthausen, Germany): nitrate, DIN EN ISO 10 304-1 (ion chromatography); sulfate, DIN EN ISO 10 304-1 (ion chromatography); ammonium, DIN 38406-E5 (colorimetry); hydrogen carbonate, DIN 38 405-D8 (titration); BTEX, DIN 38 407-F9 (GC-MS); hydrocarbon oil index, DIN EN ISO 9377-2 (solvent extraction, GC); phenol index, DIN 38 409-H16-2 (distillation, colorimetry); PAH, DIN 38 4078-F39 (GC-MS); iron, DIN EN ISO 11885 (ICP-OES). The raw data are presented in Table S1.

2.2. Cultivation of microorganisms

For enrichment of microorganisms, groundwater was sampled from well A, B, and C, filled without leaving a headspace in sterile amber stained glass bottles and stored at 4 °C in the dark. For batch cultures, 100 ml groundwater was transferred into sterile 200 ml-serum flasks. The flasks were closed with butyl rubber stoppers and residual oxygen was removed by 30 alternating cycles of evacuation and gassing with sterile-filtered nitrogen (2 min each). Afterwards, the groundwater (100 ml) was amended with 0.5 ml 1 M $NaNO_3$, 0.1 ml of a single BTEX compound (prepared as 200 mM stock

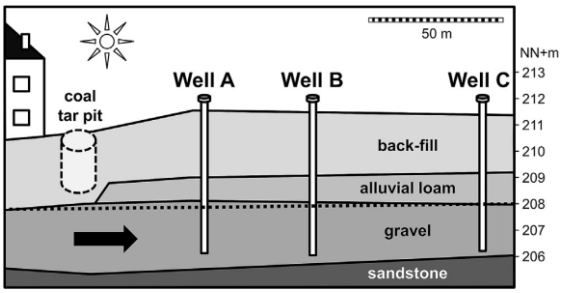

Fig. 1. Schematic cross-section of the Thuringia gasworks site showing the location of the coal tar pit and the three groundwater sampling wells A, B, and C. The dotted line indicates the water table. The arrow marks the groundwater flow direction. For further description of the geology, see section 2.1.

solutions of toluene, ethylbenzene, *p*-, *m*-, and *o*-xylene or 50 mM of benzene; each dissolved in 2,2,4,4,6,8,8-heptamethylnonane) and 1 ml of a potassium-phosphate buffered (100 mM, pH 7.5) supplement solution containing vitamins and trace metals (Mingo et al., 2016). The cultures were incubated in the dark at 24 °C with the bottleneck facing down and continuous shaking (100 rpm).

For further cultivation, an artificial mineral salt medium was used containing the following compounds (per liter ultra-pure water): 0.5 g NaCl, 0.5 g KH_2PO_4, 0.5 g $MgCl_2$ x 6 H_2O, 0.4 g NH_4Cl, 0.4 g KCl, 0.2 g $MgSO_4$ x 7 H_2O, and 0.1 g $CaCl_2$. The medium (95 ml) was transferred into 200 ml-serum flasks, which were closed with butyl rubber stoppers. Oxygen was removed by repeated degassing and flushing with nitrogen (25 cycles, 3 min each). After autoclavation, anaerobic sterile stock solutions (prepared in ultra-pure water) were added: 3 ml 1 M $NaHCO_3$ (CO_2-saturated), 0.5 ml 1 M $NaNO_3$, 0.5 ml vitamin solution, 0.1 ml SL-10 trace element solution, and 0.1 ml selenite-tungsten solution. The pH of the medium was adjusted to pH 7 by addition of 0.5 ml 1 M HCl. Single BTEX compounds (0.1 ml) were supplied from 1 M stock solutions prepared in 2,2,4,4,6,8,8-heptamethylnonane. The vitamin solution contained (per liter ultra-pure water): 8 mg *p*-aminobenzoic acid, 2 mg D(+)-biotin, 20 mg nicotinic acid, 10 mg Ca-D(+)-pantothenate, 30 mg pyridoxamine HCl, and 20 mg thiamine HCl (Pfennig, 1978; Widdel and Pfennig, 1981). The trace element solution was composed of 8.5 ml HCl (37%), 1.5 g $FeCl_2$ x 4 H_2O, 6 mg H_3BO_3, 190 mg $CoCl_2$ x 6 H_2O, 100 mg $MnCl_2$ x 4 H_2O, 70 mg $ZnCl_2$, 36 mg Na_2MoO_4 x 2 H_2O, 24 mg $NiCl_2$ x 6 H_2O, 2 mg $CuCl_2$ x 2 H_2O per liter ultra-pure water (Widdel and Pfennig, 1981; Widdel et al., 1983). The selenite-tungsten-solution contained (per liter ultra-pure water): 0.5 g NaOH, 3 mg Na_2SeO_3 x 5 H_2O, and 4 mg Na_2WO_4 x 2 H_2O. The medium was inoculated with 10 ml of a grown culture. Growth was followed by measuring the optical density at 578 nm and by determination of the protein concentration using the Roti®-Nanoquant reagent (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Bovine serum albumin (>98% purity, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was used as standard.

2.3. Analytical methods

The concentration of BTEX was determined with a Clarus 500 gas chromatograph (GC) equipped with a flame ionization detector, a Turbo Matrix 40 headspace sampler (PerkinElmer, Rodgau, Germany) and a CP-PoraBOND Q fused silica column (25 m × 0.32 mm; Agilent Technologies, Waldbronn, Germany). Nitrogen (purity 5.0; Linde AG, Pullach, Germany) was used as carrier gas (pressure 100 kPa). For headspace sampling, the GC vial containing 1 ml sample from the aqueous phase of the medium and 20 µl nonane (10 mM) as internal standard was heated for 6 min at 95 °C in the headspace sampler before injection. The temperature of the injector and detector were 250 °C and 300 °C, respectively. The following temperature program was applied for the oven: 4 min at 150 °C followed by a gradient of 10 °C/min increasing to 280 °C and hold for 4 min. The retention times were as follows: benzene, 7.5 min; toluene, 10.6 min; ethylbenzene, 13.1 min; *p*-/*m*-xylene, 13.2 min; *o*-xylene, 13.6 min; nonane, 14.2 min. Aqueous solutions of BTEX (>99% purity; Merck KGaA, Darmstadt, Germany) prepared in ultra-pure water were used as standards (25–500 µM).

Nitrate and nitrite were quantified using a high performance liquid chromatography system (Merck-Hitachi) equipped with a L-6200 pump, a 2000A autosampler, a L-4500 diode array detector and a LiChrospher® 100 RP-18 (5 µm) LiChroCART® 125-4 column (Merck KGaA, Darmstadt, Germany). As eluent, 45% (v/v) methanol plus 0.3% (v/v) H_3PO_4 in ultra-pure water was used. A flow rate of 0.4 ml min^{-1} was applied. Signals were detected at 220 nm. Under these conditions, the retention times were: nitrate, 3.0 min; nitrite, 5.2 min. Nitrate was not retained by the column and eluted together with the injection peak. The area of the injection peak was determined in controls without nitrate and subtracted from nitrate areas. The concentrations of nitrate and nitrite determined via HPLC analysis were confirmed by colorimetric assays using a resorcinol based method for nitrate (Bosch Serrat, 1998) and the Griess Reagent System for nitrite (Promega GmbH, Mannheim, Germany). Mono-element standards in the highest available purity (CPAchem, Stara Zagora, Bulgaria) were used for calibration. Ammonium was determined using the Aquaquant® ammonium test kit (Merck KGaA, Darmstadt, Germany).

2.4. DNA extraction

Groundwater samples from well A, B, and C were taken with a scoop (3 liter each), centrifuged in sterile 500 ml-polypropylene bottles (18,600x*g*, 60 min, 10 °C) and concentrated to 10 ml aqueous sludge. DNA isolation was done with the RapidWater™ DNA isolation kit (Mo Bio Laboratories, Carlsbad, USA) according to the manufacturer's protocol with some modifications: The beads for three reactions were pooled into one 5 ml reaction tube. Aqueous sludge (0.75 ml) and 3 ml of solution RW1 from the kit were added to this tube. Cells were disrupted by heating at 65 °C for 10 min followed by mixing (30 hz^{-1}, 10 min, 4 °C) with a mixer mill MM 400 (Retsch GmbH, Haan, Germany). For each groundwater sample, two preparations were done in parallel. The supernatants of the two preparations were pooled and 6 ml solution RW2 was added. The mixture was applied to a single spin filter column. Further steps were performed according to the manufacturer's manual. A negative control was conducted by using the same method of DNA extraction, but by replacing the aqueous sludge with ultra-pure water.

DNA from microbial enrichments was extracted using the innuPREP Bacteria DNA kit (Analytik Jena AG, Jena, Germany). Samples (10 ml) were taken in the late exponential growth phase and were centrifuged for 45 min (4000x*g*, 10 °C). DNA extraction was performed according to the manufacturer's protocol.

The integrity of the genomic DNA from groundwater or enrichment cultures was confirmed by gel electrophoresis (0.8% (w/v) agarose gel in 1fold TAE buffer). Quantification was done using a Qubit® fluorometer and the Qubit® dsDNA BR assay kit (Thermo Fisher Scientific GmbH, Dreieich, Germany).

2.5. Illumina sequencing of 16S rRNA

The concentration of genomic DNA in environmental samples and enrichment cultures was normalized to 10 ng/µl with microbial DNA-free water (QIAGEN GmbH, Hilden, Germany). DNA samples were sent, together with a negative control (see section 2.4), to MR DNA® (Shallowater, TX, USA) for amplicon sequencing of the 16S rRNA gene (V3/V4 region). For initial PCR amplification, the primers S-D-Bact-0341-b-S-17 (5′-CCT ACG GGN GGC WGC AG-3′) and S-D-Bact-0785-a-A-21 (5‘-GAC TAC HVG GGT ATC TAA TCC-3‘) were used (Klindworth et al., 2012), which included a sample specific barcode attached to the forward primer using the bTEFAP® service based on barcoding processes originally described in Dowd et al. (2008). A DNA library was prepared according to the Illumina TruSeq DNA library preparation protocol. Paired-end sequencing was done on an Illumina MiSeq system using the Reagent Kit v3 following the manufacturer's guidelines. For analysis of Q25 merged sequence data, the MR DNA® pipeline (Shallowater, TX, USA) was used based on suggested and default QIIME methods (Caporaso et al., 2010). It included the following steps: joining of sequences, removing of barcodes, removing of sequences that are <150 bp, removing of sequences with ambiguous base calls,

denoising, de novo picking of operational taxonomic units (OTUs) defined by 97% nucleotide identity and removing of singleton sequences and chimeras. Taxonomical classification was done using a curated in-house database derived from RDP-II (Cole et al., 2005) and NCBI (O'Leary et al., 2016). A complete list of OTUs including E-values and percent nucleotide identity to the closest match is given as Supplementary Table S2. Sequences were submitted to the European Nucleotide Archive (http://www.ebi.ac.uk/ena) and are available under the study accession number PRJEB19661.

2.6. Preparation of bamA clone libraries

The *bamA* gene was amplified from genomic DNA isolated from environmental samples and enrichment cultures using the primers oah_f (5′-GCA GTA CAA YTC CTA CAC SAC YGA BAT GGT-3′) and oah_r (5′-CCR TGC TTS GGR CCV GCC TGV CCG AA-3′) published by Staats et al. (2011). The PCR was conducted in 25 µl reactions with 25 ng DNA, 0.2 µM of each primer and 5 U Taq DNA polymerase in reaction buffer A^+ containing 1.5 mM $MgCl_2$ (segenetic, Borken, Germany). For amplification, the following conditions were applied: 94 °C for 5 min, 39 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, final elongation at 72 °C for 8 min. The PCR product was analyzed on a 2% (w/v) agarose gel prepared in 1fold TAE. The expected size of the PCR product was approximately 350 bp. The band of interest was excised and purified using the Hi Yield® Gel/PCR DNA Fragment Extraction kit (Süd-Laborbedarf GmbH, Gauting, Germany). The *bamA* gene fragment was cloned into the pCR™4-TOPO® vector using the TOPO® TA cloning® kit (Thermo Fisher Scientific GmbH, Dreieich, Germany) and transformed into *E. coli* XL 1 Blue competent cells following the heat-shock procedure (Sambrook et al., 1989). Cells were streaked on LB-agar plates containing 100 µg ml^{-1} ampicillin. The presence of correct insert was confirmed by colony PCR. Therefore, single colonies were transferred to 20 µl sterile ultra-pure water. The suspensions were incubated for 5 min at 95 °C. After centrifugation, 2 µl of supernatant was used as template in the PCR reaction. M13R (5′-CAG GAA ACA GCT ATG AC-3′) and T7 (5′-TAA TAC GAC TCA CTA TAG GG-3′) were used as primers. The annealing temperature was 55 °C and 30 cycles were performed using the conditions described above. PCR products were analyzed on a 2% (w/v) agarose gel. Transformants, which contained an insert of the correct size, were sent to GATC Biotech AG (Konstanz, Germany) for plasmid preparation and Sanger sequencing using the T7 primer. Sequence data were analyzed with the software MEGA 7 (Kumar et al., 2016) using the following procedure: orientation of sequences in the same direction, trimming of plasmid and primer derived nucleotides and a MUSCLE alignment (Edgar, 2004) with *bamA* reference sequences of cultured bacteria (for accession numbers, see Table S5). Evolutionary relationships were evaluated by the maximum likelihood method based on the general time reversible model (Nei & Kumar, 2000) with gamma distribution and invariant sites (G+I). The phylogeny was tested using the bootstrap method and 1000 replicates. Nucleotide sequence identities with *bamA* reference sequences were calculated with the 'Sequence Identity Matrix' option in BioEdit (version 7.2.5; Hall, 1999). Sequences were submitted to the European Nucleotide Archive (http://www.ebi.ac.uk/ena) and are available under the accession numbers LT800504 - LT800686 (*bamA* of groundwater samples) and LT838365 - LT838395 (*bamA* of enrichment cultures, excluding redundant sequences).

2.7. Design of degenerated primers for the amplification of 4-methylbenzoyl-CoA reductase gene fragments by PCR

A BLASTP search (Altschul et al., 1990) against metagenomes derived from hydrocarbon-/coal-tar-polluted environments (JGI IMG system; Markowitz et al., 2012) was conducted using the amino acid sequence of the 4-methylbenzoyl-CoA reductase subunit C of *Magnetospirillum* sp. pMbN1 (MbrC; GenBank accession number: AIW63090) as template. Seven putative MbrC sequences were found with an identity on protein level of 43–68% to MbrC of *Magnetospirillum* sp. pMbN1. The corresponding gene sequences (for accession numbers, see Fig. 4A) were used in an alignment to design the *mbrC* specific primers mbrc317_FW (5′-TGT TYG TYA CCC AYC CKA TCT G-3′) and mbrc1031_RV (5′-AGA CCB GGY TCR CAC ATC TTG-3′). The PCR was conducted in 25 µl reactions with 25 ng DNA, 0.4 µM of each primer and 5 U Taq DNA polymerase in reaction buffer A^+ containing 1.5 mM $MgCl_2$ (segenetic, Borken, Germany). For amplification of the *mbrC* gene fragment, the following conditions were applied: 95° for 3 min, 30 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min, final elongation at 72 °C for 10 min. The homology of the amplicon amplified from the *p*-xylene-degrading enrichment culture (~700 bp) to *mbrC* was confirmed by Sanger sequencing (GATC Biotech AG, Konstanz, Germany).

2.8. Genome walking

For genome walking, the method described for the Universal GenomeWalker™ kit (Clontech Laboratories, Mountain View, CA, USA) was followed. DNA libraries were prepared using the restriction enzymes DraI, EcoRV, and PvuII. The gene specific primers (upstream: 4MBR_GW_RV01: 5′-CCG CTC ATA CTC ATT GCG TAG-3′, 4MBR_GW_RV02: 5′-GGA TTC TGC GGA AGA TGC AG-3′; downstream: 4MBR_GW_FW01: 5′-GCT GTT GGA AGA TGT GAG CAC-3′, 4MBR_GW_FW02: 5′-GTG CAG CAC GAC AAC AAC AAG-3′) were derived from the 4-methylbenzoyl-CoA reductase gene fragment (see section 2.7). Specific PCR products of the EcoRV library were sequenced. For a second downstream walk, the primers 4MBR_GW_FW03 (5′-ATC GTC AAG CGC GAG ACC-3′) and 4MBR_GW_FW04 (5′-CTC GTA CTG CAT CAC GAC G-3′) were applied. The length of the entire fragment was 4167 bp. The open reading frames putatively encoding the subunits MbrA, MbrB, MbrC, and MbrD of a 4-methylbenzoyl-CoA reductase were aligned with reference sequences of benzoyl-CoA reductases and a 4-methylbenzoyl-CoA reductase using MUSCLE (Edgar, 2004). A phylogenetic tree was constructed using the maximum likelihood method based on the JTT matrix-based model (Jones et al., 1992) and 1000 bootstrap replicates (implemented in MEGA 7; Kumar et al., 2016). The sequence of the newly retrieved *mbrBCAD* gene cluster was deposited at the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under the accession number LT934314.

3. Results and discussion

3.1. The sampling site

The gasworks site studied here has an unsecured coal tar pit from which organic pollutants are leaking into the groundwater. A profile of the gasworks site and the location of the three groundwater sampling wells is depicted in Fig. 1. For a detailed description of the geology and the physico-chemical parameters see section 2.1 and Table S1. Median concentrations of selected groundwater parameters are given in the following: BTEX compounds were the major contaminants with highest concentrations close to the coal tar pit in well A (9.8 mg l^{-1}) and decreasing concentrations downstream in well B (1.7 mg l^{-1}) and C (0.16 mg l^{-1}). Oxygen concentrations were 0.6, 0.1, and 0.2 mg l^{-1} in well A, B, and C, respectively, reflecting the almost anoxic conditions of the aquifer. The concentration of ammonium, a waste product formed during gas production, was highest in the vicinity of the coal tar pit and

decreased further downstream (A: 23.7 mg l^{-1}, B: 14.9 mg l^{-1}, C: 5.1 mg l^{-1}). Probably due to ammonium oxidation, the concentration of nitrate was elevated in well A (13.1 mg l^{-1}) compared to B (1.4 mg ml^{-1}) and C (1.0 mg ml^{-1}). The concentration of sulfate was lowest in well A (71 mg l^{-1}) and increased further downstream (B: 317 mg l^{-1}, C: 561 mg l^{-1}), indicating sulfate-reducing conditions close to the coal tar pit (Schirmer et al., 2006). Dissolved iron, which is formed in the absence of oxygen by the reduction of insoluble Fe(III) to soluble Fe(II) was detected in well B and C (A: < 0.1 mg l^{-1}; B: 0.7 mg l^{-1}; C: 1.9 mg l^{-1}).

3.2. The microbial community of the aquifer with emphasis on aromatic compound-degrading bacteria

The microbial community of the aquifer, which was analyzed by 16S rRNA amplicon sequencing on an Illumina MiSeq platform (for a complete list of OTUs including the taxon affiliation, see Table S2; for a general summary of Illumina output data including the abundance of genera, see Table S3), was dominated by Proteobacteria with relative abundances of 78% (well A), 83% (well B) and 70% (well C), respectively (Fig. 2A). Within the Proteobacteria, the majority of sequences were classified as Betaproteobacteria (well A: 53%, well B: 35%, well C: 40%). Many Betaproteobacteria are facultative anaerobes frequently found in waste waters (Wagner et al., 2002; Nielsen et al., 2009; Wang et al., 2012). They can degrade a variety of organic compounds and seem to play a major role in the nitrate-dependent degradation of aromatic hydrocarbons (Parales, 2010). In well B, also Epsilonproteobacteria, mainly represented by *Arcobacter* spp., were highly abundant (36%). Its role in groundwater is not clear since free-living *Arcobacter* spp. have a versatile metabolism; however, they are often associated with sulfur and nitrogen cycling (Roalkvam et al., 2015). Their occurrence in contaminated environments was reported before (Gevertz et al., 2000; Collado et al., 2011; Levican et al., 2013). Other prominent phyla detected in all three groundwater wells included Actinobacteria (3.3–8.8%), Bacteroidetes (3.0–5.4%), and Firmicutes (2.8–6.7%).

The Illumina dataset was analyzed for the presence of aromatic compound-degrading bacteria (ACDB). Since the aquifer was oxygen-depleted, we focused on microorganisms, which have the potential to degrade aromatic compounds in the absence of oxygen via CoA thioesters using the benzoyl-CoA pathway (designated as oxygen-independent ACDB, short: no-ox ACDB). Furthermore, a literature search was conducted to identify taxa abundant in the aquifer that were previously associated with the degradation of aromatic compounds in oxygen-depleted environments, but for which the presence of the anaerobic benzoyl-CoA pathway was not yet described (for references and explanations, see Table S4). The majority of these taxa possess the oxygen-dependent box pathway for aromatic compound degradation as deduced from a BLAST analysis (data not shown) initially described by Rather et al. (2010). The box pathway is considered as an adaptation to low oxygen conditions (Fuchs, 2008). In this pathway, aromatic compounds are also degraded via CoA thioesters as intermediates, however, an oxygen-dependent dearomatization step is involved (for review see Fuchs et al., 2011). These taxa are designated as low-oxygen-

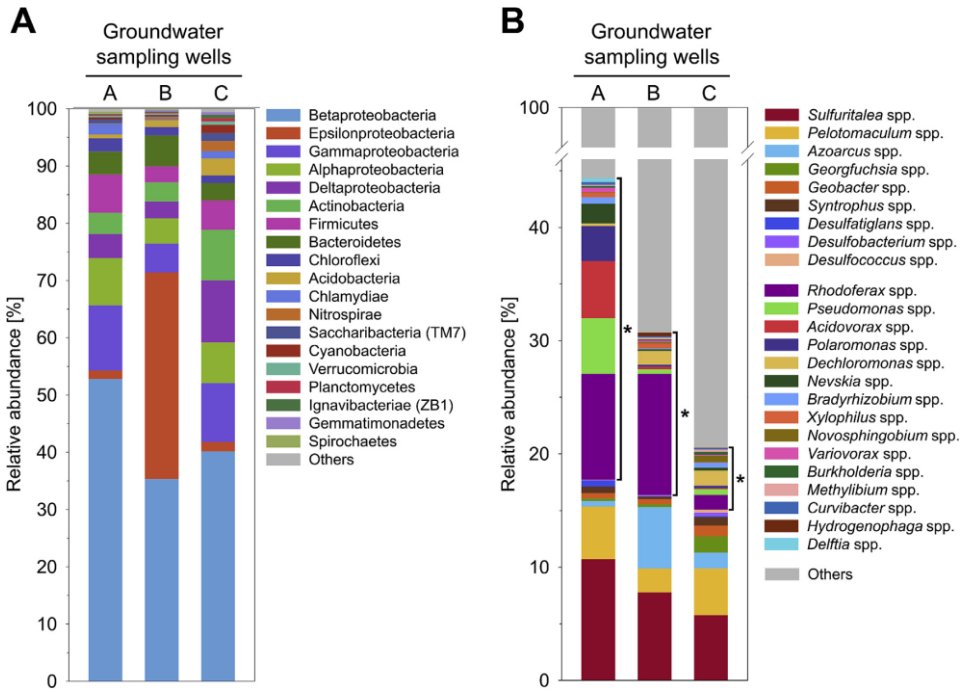

Fig. 2. (A) Relative abundance of bacterial phyla and proteobacterial classes expressed as percentage of total 16S rRNA sequences in groundwater samples. Phyla with a mean relative abundance below 0.15% were summarized as others. (B) Relative abundance of bacterial genera that were assigned as aromatic compound-degrading bacteria (ACDB) expressed as percentage of total 16S rRNA sequences in groundwater samples. ACDB with a mean relative abundance <0.1% as well as non-ACDB genera were summarized as others. The ACDB genera were sub-classified into low-oxygen-dependent (marked with an asterisk) and oxygen-independent ACDB. For explanations and references, see section 3.2 and Table S4, which also comprises the complete list of genera defined as ACDB. The data of this figure were obtained by 16S rRNA amplicon sequencing on an Illumina MiSeq platform and subsequent taxonomic classification with QIIME.

dependent ACDB (low-ox ACDB).

The highest relative abundance of total ACDB was detected in well A with 44% (Fig. 2B). The amount decreased in well B (31%) and C (21%). The number of ACDB was consistent with the concentration of BTEX in groundwater, which was highest in well A and lowest in well C. The most abundant no-ox ACDB in the aquifer were *Sulfuritalea* (well A: 10.7%, well B: 7.8%, and well C: 5.8%), *Pelotomaculum* (4.7%, 2.1%, and 4.1%), *Azoarcus* (0.5%, 5.4%, and 1.4%), *Georgfuchsia* (0.2%, 0.2%, and 1.4%), and *Geobacter* (0.5%, 0.5%, and 0.9%). The presence of *Sulfuritalea* spp. in all groundwater wells is noticeable. So far, the only pure culture of this genus is *Sulfuritalea hydrogenivorans* sk43H (Kojima and Fukui, 2011). It was isolated from a freshwater lake in Japan and is the closest relative of the strictly anaerobic ACDB *Georgfuchsia toluolica* G5G6. Aside from the ability of *S. hydrogenivorans* sk43H to degrade benzoate with nitrate (Kojima and Fukui, 2011), its role as ACDB was not investigated so far. This question will be addressed in future studies. Whereas the total amount of no-ox ACDB was roughly similar in all three sampling wells, a considerably increased abundance of low-ox ACDB including *Acidovorax, Polaromonas, Pseudomonas*, and *Rhodoferax* was observed in well A. This was in consistence with an increased oxygen concentration in this well (well A: 0.6 mg l^{-1}, well B: 0.1 mg l^{-1}, well C: 0.2 mg l^{-1}). This observation indicates that the increase of total ACDB in well A (Fig. 2B) is possibly caused by oxygen and not by BTEX concentrations. The availability of nitrate in well A (Table S1) might also positively affect the low-ox ACDB community as it was shown for *Pseudomonas sp.* (Svenningsen et al., 2016). The most abundant low-ox ACDB was *Rhodoferax* previously described to degrade benzoate with a variety of electron acceptors including Fe(III), nitrate, and oxygen (Finneran et al., 2003). Because of its versatility, the role of *Rhodoferax* in aromatic compound degradation is difficult to interpret and needs to be further elucidated. Other genera that were characteristic for the groundwater, but so far not described to degrade aromatic compounds, include *Arcobacter* (well A: 0.8%, well B: 32.4%, and well C: 0.5%), *Gallionella* (0.2%, 0.9%, and 11.3%), and *Giesbergeria* (18.3%, 0.1%, and <0.1%) (see also Table S3), all reported to commonly occur in ground- and wastewater (Rice et al., 1999; Grabovich et al., 2006; Hanert, 2006).

3.3. Application of the functional marker gene bamA

Since the taxa predicted to be involved in aromatic compound degradation based on 16S rRNA analysis do not necessarily possess the genes for biodegradation, *bamA* was applied as functional marker to target the anaerobic benzoyl-CoA pathway (Kuntze et al., 2008; 2011; Staats et al., 2011). With the *bamA* assay, no-ox ACDB are targeted, whereas low-ox ACDB cannot be detected. Fig. 3 shows a phylogenetic tree of the *bamA* nucleotide sequences recovered from clone libraries prepared with DNA from groundwater of well A, B, and C (see also Table 1). For comparison, *bamA* reference nucleotide sequences of isolated and enriched ACDB were included in the cladogram (Table S5). The obtained sequences were categorized into three clades: clade I comprises *Azoarcus*-, *Sulfuritalea*-, and *Georgfuchsia*-related *bamA* sequences. Clade II includes *Geobacter*-, *Magnetospirillum*-, and *Thauera*-related *bamA* sequences and clade III contains *bamA* sequences of various members of mainly sulfate-reducing/fermenting Deltaproteobacteria and versatile, often syntrophic Clostridia (Firmicutes). The majority of groundwater *bamA* sequences (116 of 183 clones, ≈ 63%) belonged to clade I. The *bamA* sequences within this clade were highly related to nitrate-reducing *Azoarcus* sp. EbN1 (≥93.6% identity) and *Sulfuritalea hydrogenivorans* sk43H (92.1% identity). This finding is in accordance with the 16S rRNA analysis, which revealed a high abundance of the no-ox ACDB-genera *Azoarcus* and *Sulfuritalea* in groundwater of the aquifer. Clade II includes 39 *bamA* sequences. A single sequence within this clade was related to *Magnetospirillum* sp. pMbN1 (94.2% identity). The remaining sequences either belonged to a cluster related to Fe(III)-reducing *Geobacteraceae* (86.5–88.5% identity), which were also identified by the 16S rRNA analysis, or showed similarity to phototrophic and nitrate-reducing *Alphaproteobacteria* (83.5–86.5% identity). The 28 sequences affiliated to clade III originated, with one exception, from sulfate-depleted groundwater of well A. These sequences were similar to sulfate-reducing/fermenting Deltaproteobacteria such as *Desulfatiglans anilini* (80.2% identity) and *Desulfomonile tiedjei* (77.1% identity) or were affiliated to syntrophic Firmicutes related to *Desulfotomaculum* sp. BICA1-6 (78.8% identity). Low similarities to reference sequences indicate the presence of so far uncharacterized members of Deltaproteobacteria and Firmicutes. The increased occurrence of these obligate anaerobic bacteria in well A was not apparent from analyzing the 16S rRNA sequences: the only dominant genus, which would group into clade III identified by 16S rRNA analysis was *Pelotomaculum*, however, 16S rRNA sequences affiliated to this genus were present in all sampling wells instead of being restricted to well A. This example highlights the advantage of combining 16S rRNA profiling with *bamA* analysis: while 16S rRNA sequencing allows a refined taxonomic classification, *bamA* is superior in identifying microbial degradation processes, in this case a sulfate-reducing/fermenting ACDB community in sulfate-depleted groundwater.

The *bamA* gene was also used by other researchers to target ACDB, but a deep 16S rRNA-based community profiling was not performed in these studies (e.g. Kuntze et al., 2011; Staats et al., 2011; Porter and Young, 2013; Verde et al., 2013; Sun et al., 2014b; Ruan et al., 2016). Sampling sites that were analyzed included contaminated soil or sludge, deep subsurface oil fields, and BTEX contaminated aquifers. Each site (or sample) was characterized by a specific '*bamA* community'. Some of the researchers suggested that the electron acceptor available in the habitat or sample had a major influence on the composition of the '*bamA* community'. According to this, the iron-reducing Geobacteraceae were the most abundant ACDB in the iron-containing aquifer analyzed by Staats et al. (2011). Strictly anaerobic Deltaproteobacteria and/or Firmicutes dominated the microbial communities in production waters of deep subsurface oil fields, where the presence of oxygen is unlikely (Verde et al., 2013; Ruan et al., 2016). The aquifer of the Thuringia gasworks site we have analyzed in this study was dominated by *bamA* sequences of presumably nitrate-reducing Betaproteobacteria indicating the importance of nitrate as electron acceptor for aromatic compound degradation at this site. This finding has considerable implications for the design of future bioremediation strategies and suggests the infiltration of nitrate as suitable approach to promote the indigenous microbial community in aromatic compound degradation. Previously, nitrate was successfully applied by other researchers as stimulating agent for *in situ* bioremediation (Cunningham et al., 2001; Eckert and Appelo, 2002; Rivett et al., 2008; Xu et al., 2014; 2015). Besides the occurrence of nitrate-reducing Betaproteobacteria in all sampling wells, additional *bamA* sequences affiliated to strictly anaerobic Deltaproteobacteria and Firmicutes were present in the aquifer, but mainly restricted to the highly contaminated, sulfate-depleted well A. These strictly anaerobic microorganisms are often involved in sulfate reduction or fermentation. The utilization of sulfate as electron acceptor would explain the observed decrease in the sulfate concentration in well A. In addition, well A was characterized by an increased abundance of low-ox ACDB, which require at least small amounts of oxygen for aromatic compound degradation (see Fig. 2B). At a first glance, the presence of nitrate-reducing, strictly anaerobic and low-ox ACDB in the same well is

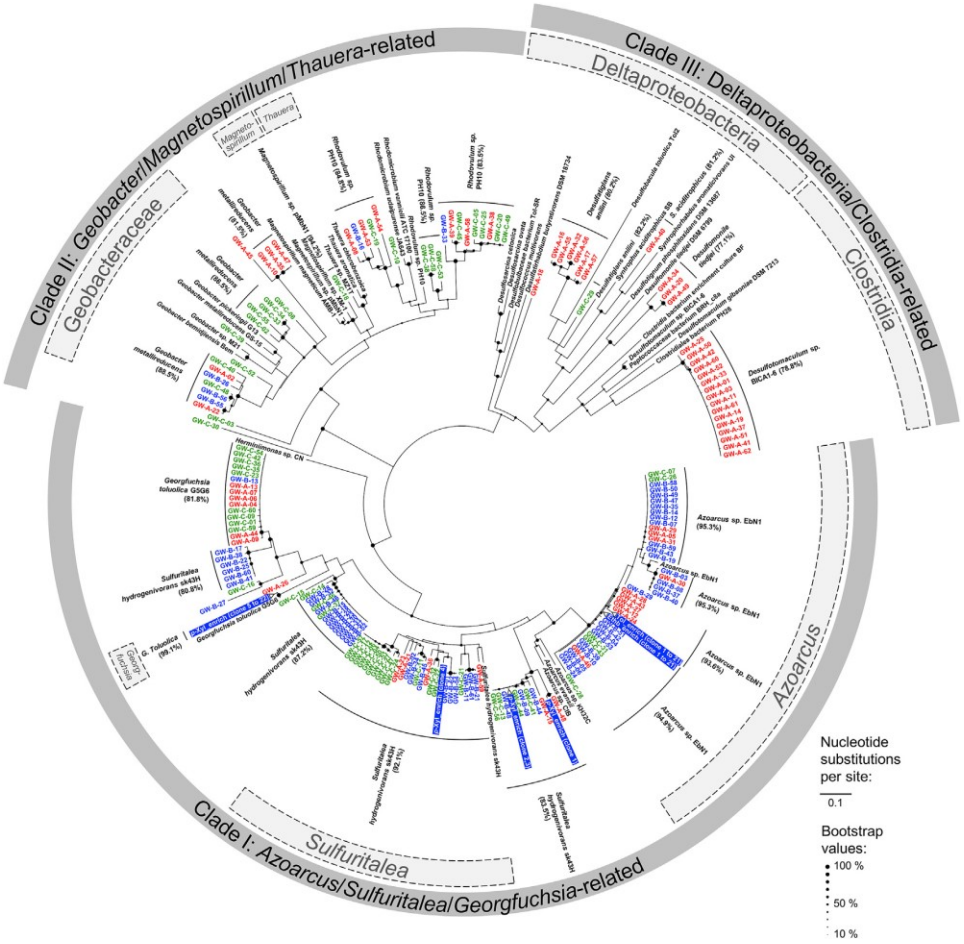

Fig. 3. Phylogenetic tree of *bamA* gene fragments constructed by using the maximum likelihood method based on the general time reversible model. The *bamA* nucleotide sequences were retrieved from groundwater samples of well A (red, GW-A-no.), well B (blue, GW-B-no.), and well C (green, GW-C-no.) as well as from nitrate-reducing enrichment cultures (highlighted in blue) utilizing toluene (Tol_enrich), ethylbenzene (Ethb_enrich) or *p*-xylene (*p*-Xyl_enrich) as electron donor. For classification, *bamA* nucleotide sequences of known aromatic compound-degrading bacteria (ACDB) were included in the cladogram. The *bamA* sequences within the three clades were further assigned to specific groups of ACDB based on nucleotide identities to reference sequences (given as median values in parentheses). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

contradictory to the assumption that contaminant plumes are separated into distinct redox zones (Lovley, 1997). However, recent findings indicate that different redox processes can occur in parallel, especially close to the source of contamination at the plume fringe, where no limitation of the electron donor is expected (Meckenstock et al., 2015). The excess of electron donor in well A would also explain why microorganisms using energetically less favorable electron acceptors such as sulfate or CO_2 can compete in this well against microorganisms that utilize e.g. oxygen, nitrate or iron. In conclusion, our results provide further evidence that *bamA* profiling is a valuable method to predict electron-accepting processes in environmental samples and a future application is suggested to assist in the design and/or monitoring of bioremediation approaches, preferentially by the use of next-generation sequencing techniques.

3.4. Groundwater enrichment cultures

The involvement of nitrate-reducing ACDB in the degradation of aromatic compounds at the site was verified by cultivation attempts. Anoxic groundwater of well A, B, and C was amended with nitrate as electron acceptor and single BTEX compounds as electron donor. Under the conditions applied, ethylbenzene, toluene, *p*- and *m*-xylene were degraded (Table 2). The consumption rates and the lag phases differed among the BTEX tested and the groundwater wells used for sampling (Table 2). Toluene and ethylbenzene were degraded fastest in all groundwater samples with consumption rates ranging from 4 to 7 μmol d^{-1}. The degradation started either immediately (well B) or after 4–7 days of incubation (wells A and C). For *p*- and *m*-xylene, consumption started after prolonged lag phases of 2 and 5 weeks in groundwater obtained from well A and B, respectively. The average consumption rate was 1.4 μmol d^{-1} for *p*-xylene and 0.6 μmol d^{-1} for *m*-xylene. Higher consumption rates

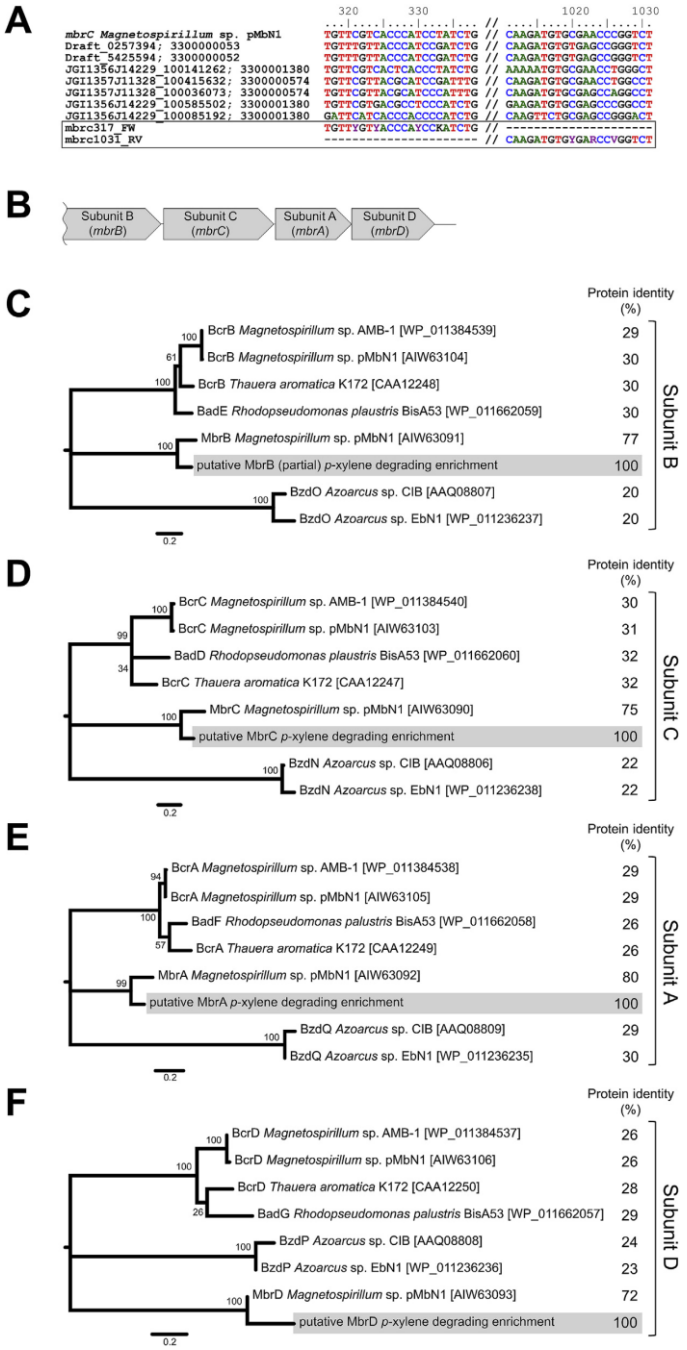

Fig. 4. Identification of a gene region encoding a putative 4-methylbenzoyl-CoA reductase (*mbrBCAD*) in the *p*-xylene-degrading enrichment culture. (A) Sequence alignment of *mbrC* of *Magnetospirillum* sp. pMbN1 with seven putative *mbrC* genes retrieved from metagenomic databases (IMG Gene ID and IMG Genome ID given in the figure) and the *mbrC*-specific degenerated primer sequences used for amplification of the respective gene fragment. The nucleotide numbers indicated are based on *mbrC* of *Magnetospirillum* sp. pMbN1. (B) The putative *mbrBCAD* gene-cluster of the *p*-xylene-degrading enrichment culture. The gene organization is identical to *Magnetospirillum* sp. pMbN1. For subunit B, only a partial gene region was obtained by genome walking. (C-F) Phylogenetic trees showing the relatedness of the four putative subunits MbrA-D from the *p*-xylene-degrading enrichment culture (EMBL accession number: LT934314) to MbrA-D of *Magnetospirillum* sp. pMbN1 as well as to corresponding subunits of several characterized benzoyl-CoA reductases (BcrA-D, BadD-G, BzdN-Q) from model organisms. Protein sequences were aligned with MUSCLE and the trees were constructed using the maximum likelihood method, both implemented in MEGA 7.

Table 1
Relative abundance in percent and absolute number (in parenthesis) of affiliated *bamA* nucleotide sequences obtained from clone libraries prepared with DNA isolated from groundwater of well A, B, and C as well as from enrichment cultures of well B amended with nitrate as electron acceptor and either toluene (Tol), ethylbenzene (Ethb) or *p*-xylene (*p*-Xyl) as electron donor. The taxonomic affiliation is based on clustering and nucleotide identity of obtained *bamA* sequences with *bamA* reference sequences (see also Fig. 3).

Affiliated clades	Groundwater well			Enrichment culture		
	Well A	Well B	Well C	+Tol	+Ethb	+*p*-Xyl
Clade I	**37.1** (23)	**91.8** (56)	**61.7** (37)	**100** (21)	**100** (21)	**100** (22)
Azoarcus	16.1 (10)	41.0 (25)	6.7 (4)	100 (21)	100 (21)	0 (0)
Sulfuritalea	6.5 (4)	14.8 (9)	23.3 (14)	0 (0)	0 (0)	4.5 (1)
Georgfuchsia	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	81.8 (18)
unclassified	14.5 (9)	36.1 (22)	31.7 (19)	0 (0)	0 (0)	13.6 (3)
Clade II	**19.4** (12)	**8.2** (5)	**36.7** (22)	**0.0** (0)	**0.0** (0)	**0.0** (0)
Geobacteraceae	3.2 (2)	4.9 (3)	15.0 (9)	0 (0)	0 (0)	0 (0)
Magnetospirillum	0 (0)	0 (0)	1.7 (1)	0 (0)	0 (0)	0 (0)
unclassified	16.1 (10)	3.3 (2)	20.0 (12)	0 (0)	0 (0)	0 (0)
Clade III	**43.5** (27)	**0.0** (0)	**1.7** (1)	**0.0** (0)	**0.0** (0)	**0.0** (0)
Deltaproteobacteria	17.7 (11)	0 (0)	1.7 (1)	0 (0)	0 (0)	0 (0)
Clostridia	25.8 (16)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Table 2
Consumption of BTEX in groundwater samples originating from well A, B, and C using nitrate as electron acceptor.

BTEX	Well A		Well B		Well C	
	lag phase[a] [d]	consumption rate[b] [μmol d^{-1}]	lag phase [d]	consumption rate [μmol d^{-1}]	lag phase [d]	consumption rate [μmol d^{-1}]
Benzene	>140	0	>140	0	>140	0
Toluene	7	5	<1	5	7	4
Ethylbenzene	7	5	<1	7	4	4
o-Xylene	>140	0	>140	0	>140	0
m-Xylene	25	0.5	28	0.6	>140	0
p-Xylene	12	1.5	28	1.3	>140	0

[a] Days before first observation of BTEX degradation and nitrate reduction.
[b] Average consumption rate of BTEX calculated between first observation of BTEX utilization and its complete degradation.

for *p*-xylene than for *m*-xylene are noteworthy, since earlier studies implied that *p*-xylene and other *p*-alkylated monoaromatics are particularly recalcitrant in comparison to their *meta*- or *ortho*-substituted homologs (Rabus et al., 1996; 1999; Wilkes et al., 2000). The nitrate/BTEX-treated groundwater of well B was used as inoculum to obtain stable enrichment cultures in a defined mineral salt medium amended with nitrate and either toluene, ethylbenzene, *p*- or *m*-xylene. Enrichment cultures were obtained with toluene, ethylbenzene and *p*-xylene (for representative growth curves and microphotographs, see Fig. S1). In the presence of *m*-xylene, no stable culture was achieved, possibly caused by the accumulation of nitrite or toxic *m*-xylene concentrations. The 16S rRNA and *bamA* profiling revealed that toluene and ethylbenzene favored the enrichment of *Azoarcus* species (analyzed after 14 transfers into fresh medium): the relative abundance of *Azoarcus*-affiliated 16S rRNA sequences increased approximately 16fold from 5.4% in groundwater to about 85% in the enrichment cultures (see Table S3). In accordance, all *bamA* gene fragments recovered from these enrichment cultures showed highest similarity to *Azoarcus* sp. EbN1 (Fig. 3; sequences designated as Tol/Ethb_enrich highlighted in blue). In groundwater of well B, which was used for the enrichment, *Azoarcus* spp. showed a considerably higher abundance than in the other two wells. This result, in combination with the observation that the genus *Azoarcus* seems to be adapted to the

degradation of toluene and ethylbenzene, led to the assumption that well B is a hotspot of *Azoarcus*-dependent toluene and ethylbenzene degradation. This finding also explains the enrichment of *Azoarcus*-dominated cultures without lag phases from this well (Table 2). In contrast, the *p*-xylene-degrading enrichment culture (analyzed after 5 transfers into fresh medium) was dominated by *Georgfuchsia* spp. with a relative abundance of 60.8% of total 16S rRNA sequences (see Table S3). This corresponds to a 250fold enrichment compared to its abundance in groundwater of well B (0.24%). Also *Azoarcus* (4.4%), *Sulfuritalea* (3.8%), *Geobacter* (2.8%), and *Rhodoferax* (1.1%) were detected, however, relative abundances were similar to those initially detected in groundwater samples. In accordance to 16S rRNA-based community analysis, the majority of *bamA* gene fragments retrieved from the *p*-xylene-degrading enrichment culture were closely related to *Georgfuchsia toluolica* G5G6 (99.1% identity; Fig. 3, sequences designated as *p*-Xyl_enrich (clones 5 to 22) highlighted in blue). *Georgfuchsia*-related 16S rRNA sequences were considerably less abundant in the aquifer than *Azoarcus*-related sequences (Fig. 2B). For *bamA*, *Georgfuchsia*-related sequences were even below the detection limit (Table 1). This suggests that the here enriched *Georgfuchsia* sp. is rather rare in the aquifer, which also explains the prolonged lag phases observed for *Georgfuchsia*-dominated enrichments with *p*-xylene (Table 2).

3.5. Identification of a putative 4-methylbenzoyl-CoA reductase in the p-xylene-degrading enrichment culture

Whereas the genus *Azoarcus* is well-known for the degradation of toluene and ethylbenzene under denitrifying conditions (e.g. Fries et al., 1994; Rabus and Widdel, 1995; Zhou et al., 1995; Ball et al., 1996), *Georgfuchsia* spp. were so far not reported to degrade *p*-xylene. The only isolated representative within this genus, *Georgfuchsia toluolica* G5G6, was described to grow with ethylbenzene and toluene, but no growth was observed with *p*-xylene when nitrate was applied as electron acceptor (Weelink et al., 2009). Under the conditions we applied, *p*-xylene degradation was coupled to the reduction of nitrate to nitrite (Fig. S1). As reported for the type strain, in our study nitrate could be replaced by Fe(III) as electron acceptor (data not shown). The ability of the enriched *Georgfuchsia* sp. to degrade *p*-xylene suggests the presence of a novel type of class I benzoyl-CoA reductase specific for aromatic ring reduction of the *p*-methylated benzoyl-CoA intermediate previously found in the Alphaproteobacterium *Magnetospirillum* sp. pMbN1 (Lahme et al., 2012). To check for the presence of genes putatively encoding this novel type of enzyme, degenerated primers targeting the 4-methylbenzoyl-CoA reductase subunit C (*mbrC*) were developed and applied on DNA of the *p*-xylene-degrading enrichment culture. A ~700 bp fragment was amplified. The translated protein sequence showed 75% identity to the MbrC subunit of *Magnetospirillum* sp. pMbN1. Genome walking was used to identify the gene region containing *mbrA*, *mbrB*, *mbrC*, and *mbrD*. The results are shown in Fig. 4. All subunits showed highest protein identity (72–80%) to the corresponding Mbr subunits of *Magnetospirillum* sp. pMbN1. The identity to conventional class I benzoyl-CoA reductase subunits was equal or below 32%. These data indicate the presence of a benzoyl-CoA reductase specific for *p*-alkylated substrates in the *p*-xylene-degrading enrichment culture dominated by *Georgfuchsia* sp. However, it cannot be excluded that other genera present in this culture (e.g. *Sulfuritalea*, *Geobacter*) are involved in the degradation of *p*-xylene. The occurrence of a specific 4-methylbenzoyl-CoA reductase is rare in so far characterized ACDB (Rabus et al., 2016). Nevertheless, the genetic potential to utilize *p*-alkylated aromatic compounds is established at the Thuringia gasworks site, which is supported by higher degradation rates observed for *p*-xylene than for *m*- and *o*-xylene (Table 2). So far, a few enrichment cultures have been described to degrade *p*-xylene under different electron-accepting conditions (Häner et al., 1995; Morasch and Meckenstock, 2005; Nakagawa et al., 2008; Rotaru et al., 2010). Among these, a highly enriched, nitrate-reducing culture was described, which was dominated by a *Denitratisoma*-related phylotype, a close relative of *Georgfuchsia toluolica* G5G6 (Rotaru et al., 2010). The only isolated *p*-xylene-degrading bacterium reported so far uses sulfate as electron acceptor and is related to the genus *Desulfosarcina* (Deltaproteobacteria) (Higashioka et al., 2012). Our study provides a new *p*-xylene-degrading enrichment culture dominated by *Georgfuchsia* sp. Ongoing studies aim for the isolation of the microorganism(s) responsible for the degradation of *p*-xylene. This might allow further insights into the pathways involved in the anaerobic degradation of *p*-xylene and other *p*-alkylated monoaromatics, a microbial feature which is still to date rarely reported.

4. Conclusion

Deep 16S rRNA and *bamA* clone library sequencing were combined to characterize the microbial community of a coal tar polluted aquifer with emphasis on aromatic compound-degrading bacteria. The importance of nitrate as electron acceptor for aromatic compound degradation at the site was confirmed by the enrichment of nitrate-reducing toluene-, ethylbenzene- and *p*-xylene-degrading microorganisms from groundwater of the gasworks site. A close relative of *Georgfuchsia toluolica* G5G6 was found to be involved in the degradation of *p*-xylene, a compound, which is rarely observed to be degraded under anoxic conditions. The methods applied here allowed the identification of hotspots for aromatic compound degradation and the prediction of the prevailing electron-accepting processes in the aquifer, which are valuable information to predict and/or monitor the outcome of bioremediation.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2017.12.040.

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2.3 MANUSCRIPT II

Title: Anaerobic aromatic compound degradation in Sulfuritalea hydrogenivorans sk43H

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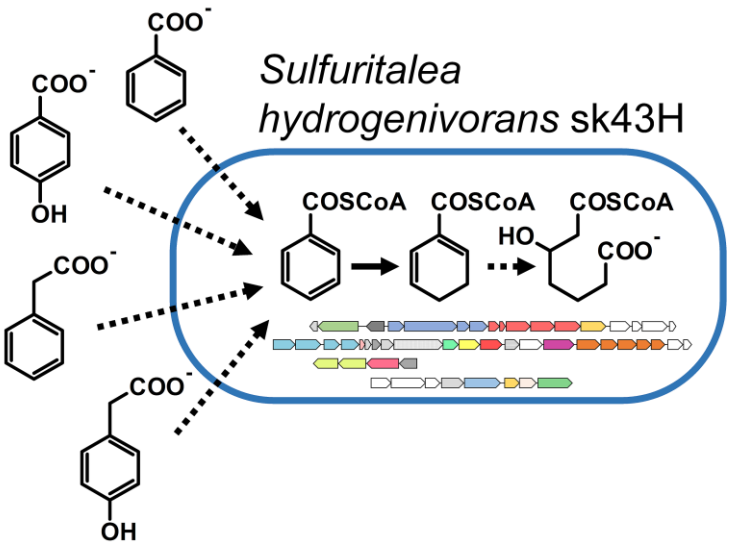
Supplementary materials: see chapter V, p. xii - xvi

Abstract:

Sulfuritalea hydrogenivorans sk43H is well-recognized as a chemolithoautotrophic microorganism that oxidizes thiosulfate, sulfur or hydrogen. In this study, pathways for aromatic compound degradation were identified in the respective genome and proved for functionality by cultivation. *S. hydrogenivorans* sk43H harbors gene clusters encoding pathways for the anaerobic degradation of benzoate and phenylacetate via benzoyl-CoA as well as a partial pathway for anaerobic cinnamate degradation. Aerobic hybrid pathways were identified for the degradation of benzoate and 2-aminobenzoate. An aerobic pathway involving mono- and dioxygenases was found for 4-hydroxybenzoate. The organization of the gene clusters for anaerobic aromatic compound degradation in *S. hydrogenivorans* sk43H was found to be similar to that of the corresponding gene clusters in "*Aromatoleum aromaticum*" strain EbN1. Cultivation experiments revealed that *S. hydrogenivorans* sk43H degrades benzoate, 4-hydroxybenzoate, phenylacetate, and 4-hydroxyphenylacetate under nitrate-reducing conditions. The results imply a so far overlooked role of this microorganism in anaerobic aromatic compound degradation, e.g. at hydrocarbon-polluted sites.



Introduction

Members of the genus *Sulfuritalea* are well recognized as sulfur-oxidizing bacteria that can grow under chemolithoautotrophic conditions (Kojima & Fukui, 2011; Watanabe *et al.*, 2014; Herrmann *et al.*, 2017). Up to now, the only pure-culture obtained is the facultative anaerobic type-strain *S. hydrogenivorans* sk43H (class Betaproteobacteria). This organism was isolated from the hypoxic hypolimnion of a stratified fresh-water lake in Japan with thiosulfate as electron donor, nitrate as electron acceptor and CO_2 as sole carbon source (Kojima & Fukui, 2011). Further electron donors utilized are elemental sulfur, hydrogen, and short-chain organic carboxylates. Pyruvate fermentation was also reported. Alternative electron acceptors are oxygen or arsenate (Watanabe *et al.*, 2017). Uncultivated *Sulfuritalea* spp. are frequently detected in fresh-water habitats, low in carbon, where they seem to play an important role in sulfur oxidation and nitrate reduction (Biderre-Petit *et al.*, 2011; Ferrer *et al.*, 2011; Kojima *et al.*, 2014; Lau *et al.*, 2016; Zeng *et al.*, 2016; Watanabe *et al.*, 2016 & 2017; Herrmann *et al.*, 2017). In addition, *Sulfuritalea*-related organisms were detected in carbon-rich habitats such as activated sludge (Juretschko *et al.*, 2002; McIlroy *et al.*, 2015), hydrocarbon-polluted sites (Yagi *et al.*, 2010; Martin *et al.*, 2012; Guo *et al.*, 2017; Sperfeld *et al.*, 2018) or aromatic compound-degrading anaerobic enrichment cultures (Rotaru *et al.*, 2010; Keller *et al.*, 2017; Sperfeld *et al.*, 2018). Its function in the latter habitats remains to be elucidated. Due to its close relatedness to aromatic-compound degrading bacteria (ACDB) (Kojima & Fukui, 2011), an involvement of *Sulfuritalea* spp. in the degradation of aromatics is conceivable. One strategy to identify ACDB in environmental samples is the detection of conserved genes of the anaerobic benzoyl-CoA pathway such as *bamA*, which encodes a ring-cleaving hydrolase (for review see von Netzter *et al.*, 2016). A re-analysis of published *bamA* sequences of environmental samples (Staats *et al.*, 2011; Kuntze *et al.*, 2011; Anantharaman *et al.*, 2016) as well as recent *bamA* studies (Sampaio *et al.*, 2017: Sperfeld *et al.*, 2018) revealed a frequent detection of *Sulfuritalea*-related *bamA* sequences in carbon-rich habitats (Tab. S1). These findings indicate a possible involvement of these microorganisms in the anaerobic degradation of aromatic compounds.

For the type-strain *S. hydrogenivorans* sk43H, growth with the aromatic compound benzoate was observed under denitrifying conditions, however, substrate-conversion rates were not reported and PCR amplification of marker genes of the anaerobic benzoyl-CoA pathway failed (Kojima & Fukui, 2011). Despite the observation that about 5% of the genes identified in *S. hydrogenivorans* sk43H showed highest similarity to genes of the aromatic compound degrader "*Aromatoluem aromaticum*" strain EbN1 (Table S2 in Watanabe *et al.*, 2014), the genome of *S. hydrogenivorans* sk43H was so far not analyzed for pathways

involved in aromatic compound degradation. Hence, it is no surprise that a possible contribution of *Sulfuritalea* spp. in this metabolic trait is rarely discussed.

This study provides a genetic and physiological basis for aromatic compound degradation in *S. hydrogenivorans* sk43H. We re-analyzed the genome sequence of the type-strain for the presence of pathways putatively involved in aromatic compound degradation and tested different monoaromatics as growth substrates applying nitrate-reducing conditions. The aim was to emphasize the potential role of *Sulfuritalea* spp. in anaerobic aromatic compound degradation besides its already well-recognized role as sulfur-oxidizing bacterium.

Materials and Methods

Cultivation of Sulfuritalea hydrogenivorans *sk43H*

S. hydrogenivorans sk43H (DSM-22779) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cultivation was carried out in 50 ml-serum flasks in the dark under continuous shaking (100 rpm) with the bottleneck facing down at 24°C. Serum flasks were filled with 25 ml of an anaerobic mineral salt medium described in Sperfeld *et al.* (2018) with slight modifications: the vitamin stock solution further contained vitamin B_{12}, riboflavin, folic acid, and α-lipoic acid (10 mg/l each). The following aromatic substrates were prepared in ultra-pure water (25 mM each, pH 7, adjusted with NaOH): anthranilate, 4-aminobenzoate, benzoate, cinnamate, 4-ethylbenzoate, 4-hydroxybenzoate, 4-hydroxycinnamate, 4-hydroxyphenylacetate, 4-isopropylbenzoate, 2-methylbenzoate, 3-methylbenzoate, 4-methylbenzoate, 4-methylcinnamate, 4-methylphenylacetate, nicotinate, phenylacetate, phenylalanine and *p*-cresol. For toluene and *p*-xylene, stock solutions (250 mM each) were prepared in 2,2,4,4,6,8,8-heptamethylnonane. For cultivation, media were amended with 7.5 mM nitrate and 1 mM of a single aromatic compound. Growth was assessed by visual observation of the turbidity (OD_{max} at 578 nm ~0.15) and bright-field microscopy.

Analytical methods

Nitrate, nitrite, and polar aromatic compounds were quantified using a high performance liquid chromatography system (Merck-Hitachi) equipped with a LiChrospher® 100 RP-18 (5 mm) LiChroCART® 125-4 column (Merck KGaA, Darmstadt, Germany). A flow rate of 0.4 ml min^{-1} was applied and signals were detected at 220 nm. Methanol in 0.3% (v/v) H_3PO_4 was used as eluent applying the following gradient: 5% (v/v) methanol (0 - 2 min), increase to 70% (v/v) methanol (2 - 9 min), hold 70% (v/v) methanol (9 - 19 min), decrease to 5% (v/v) methanol (19 - 20 min), hold 5% (v/v) methanol (20 - 40 min). Retention times of analytical standards were as follows: nitrate (3.2 min), nicotinate (4.7 min), 4-

aminobenzoate (5.9 min), nitrite (7.2 min), 4-hydroxyphenylglyoxylate (8.5 min), anthranilate (10.4 min), phenylalanine (10.5 min), phenylglyoxylate (12.5 min), 4-hydroxyphenylacetate (13.6 min), 4-hydroxybenzoate (14.0 min), 4-hydroxycinnamate (15.4 min), phenylacetate (15.8 min), *p*-cresol (16.4 min), benzoate (16.7 min), 2-methylbenzoate (17.2 min), 3-methylbenzoate (17.6 min), cinnamate (17.7 min), 4-methylphenylacetate (17.7 min), 4-methylbenzoate (18.4 min), 4-ethylbenzoate (19.6 min), 4-methylcinnamate (21.1 min), and 4-isopropylbenzoate (22.0 min). The formation of phenylglyoxylate and 4-hydroxyphenylglyoxylate as intermediates during cultivation was confirmed by addition of analytical standards to the respective culture samples. Toluene and *p*-xylene were analyzed by headspace gas chromatography according to Sperfeld *et al.* (2018).

Bioinformatic analysis

The genome of *S. hydrogenivorans sk43H* (GenBank accession number: AP012547.1) was analyzed for candidate pathways involved in the anaerobic degradation of aromatic compounds using SEED Viewer (Overbeek *et al.*, 2005). Genome comparison of *S. hydrogenivorans* sk43H and "*Aromatoleum aromaticum*" strain EbN1 (GenBank accession number: CR555306.1) was performed with the sequence-based comparison tool implemented in SEED Viewer.

Results

Candidate pathways for the anaerobic degradation of aromatic compounds

Re-analysis of the genome of *Sulfuritalea hydrogenivorans* sk43H revealed the presence of gene clusters encoding complete pathways for the anaerobic degradation of phenylacetate and benzoate via benzoyl-CoA as well as a partial pathway for anaerobic cinnamate degradation. Genes of an aerobic benzoate hybrid pathway were also identified. The organization of the gene clusters encoding these pathways is similar to that of the corresponding gene clusters of "*Aromatoleum aromaticum*" strain EbN1; in addition a high nucleotide sequence identity to corresponding genes of strain EbN1 was observed (Tab. S2). In the following, the candidate pathways identified in the genome of *S. hydrogenivorans* were compared with those encoded in "*A. aromaticum*" strain EbN1.

*Anaerobic degradation of benzoate and the upper benzoyl-CoA pathway (*bcr/bzd *gene cluster)*

In general, the anaerobic degradation of monoaromatics can be divided into three steps: (i) a peripheral 'activation' pathway resulting in the formation of benzoyl-CoA or its analogues,

(ii) the upper benzoyl-CoA pathway, which starts with the reductive dearomatization and ends up with the cleavage of the ring structure, and (iii) the lower benzoyl-CoA pathway resulting in the formation of acetyl-CoA and CO_2 (for review see Carmona *et al*., 2009). The *bcr*/*bzd* cluster found in *S. hydrogenivorans* sk43H harbors the genes encoding the enzymatic reactions of step (i) and (ii) as well as an ABC transport system (*SUTH_01660-01664*) putatively involved in the ATP-dependent uptake of benzoate (Fig. 1A). An additional benzoate-H^+ symporter (*benK*, *ebA5311*), which is present in "*A. aromaticum*" strain EbN1, was not detected. In *S. hydrogenivorans* sk43H, benzoate is probably activated by a benzoate-CoA ligase (*SUTH_01659*) forming benzoyl-CoA. This central intermediate is channeled into the upper benzoyl-CoA pathway starting with the reductive dearomatization of benzoyl-CoA catalyzed by a class I benzoyl-CoA reductase (*bcrCBDA*, *SUTH_01645-01648*). In the genome of *S. hydrogenivorans* sk43H, a single benzoyl-CoA reductase is encoded, whereas two additional reductases are present in "*A. aromaticum*" strain EbN1. These additional reductases seem to be specific for 3- and 4-hydroxybenzoyl-CoA, respectively (Wöhlbrand *et al*., 2007). After dearomatization, degradation continues via modified β-oxidation involving a dienoyl-CoA hydratase, a hydroxyacyl-CoA dehydrogenase and a ring-opening hydrolase most probably encoded by *SUTH_01654* (*dch*), *SUTH_01655* (*had*), and *SUTH_01656* (*oah*), respectively. The latter gene is also known as *bamA* and used as functional gene marker to target microorganisms harboring the anaerobic benzoyl-CoA pathway (e.g. Kuntze *et al*., 2008; Staats *et al*., 2011; Ruan *et al*., 2016). The reaction product of the ring-opening hydrolase is further metabolized by reactions of the lower benzoyl-CoA pathway, which is described in more detail later in the text. The *SUTH_1658* gene most probably encodes a glutaryl-CoA dehydrogenase, which is a key enzyme of this lower benzoyl-CoA pathway and part of the *bcr*/*bzd* gene cluster. This is not common among the well-studied aromatic compound degrading (facultative) anaerobes (for review see Carmona *et al*., 2009). In addition, a putative thioesterase gene (*SUTH_01666*) is present at the 3-prime end of the *bcr*/*bzd* cluster. This thioesterase might be involved in CoA salvaging under unfavorable conditions (Song *et al*., 2006). The *bzdR* gene (*ebA5278*) putatively encoding a transcriptional regulator in "*A. aromaticum*" strain EbN1 is absent in the *bcr*/*bzd* gene cluster of *S. hydrogenivorans* sk43H.

*The aerobic benzoate hybrid pathway (*box *gene cluster)*

A second pathway for the degradation of benzoate, the so-called aerobic benzoate hybrid pathway, is also encoded in the genome of *S. hydrogenivorans* sk43H. This alternative route to the strictly anaerobic benzoyl-CoA dearomatization involves a benzoyl-CoA oxygenase (*boxAB*, *SUTH_01641* & *01642*) that requires one molecule of oxygen. The reaction results in the formation of an epoxide (Rather *et al.*, 2010). Subsequent ring-cleavage is catalyzed

by a benzoyl-CoA dihydrodiol lyase (*boxC*, *SUTH_01643*). The *box* gene cluster of *S. hydrogenivorans* sk43H, which also harbors a putative transcriptional regulator (*SUTH_01644*), is located on the reverse strand, directly upstream of the cluster encoding the anaerobic benzoyl-CoA pathway (Fig. 1B). An additional benzoate-CoA ligase (*bclA*), which is often conserved in *box* gene clusters (Valderrama *et al.*, 2012) as well as other accessory proteins that are present in the *box* gene cluster of "*A. aromaticum*" strain EbN1, were not found in *S. hydrogenivorans* sk43H.

Another hybrid pathway (encoded by *SUTH_01525-01527*) involving a CoA-bound intermediate might be involved in the utilization of 2-aminobenzoate. This pathway was previously studied in *Azoarcus evansii* (Schühle *et al.*, 2001). The gene cluster in *S. hydrogenivorans* sk43H encodes a putative anthraniloyl-CoA monooxygenase (*SUTH_01524*), two enzymes possibly involved in β-oxidation (*SUTH_01525*, *SUTH_01526*), and a putative regulator (*SUTH_01527*). A specific CoA ligase for the initial activation of 2-aminobenzoate to 2-aminobenzoyl-CoA is not encoded in the cluster, however, it is proposed that a regular benzoate CoA-ligase might also recognize 2-aminobenzoate (Schühle *et al.*, 2003).

*Anaerobic peripheral pathway of phenylacetate conversion (*pad *gene cluster)*

The conversion of phenylacetate into the central intermediate benzoyl-CoA proceeds in three steps: (i) activation of phenylacetate to phenylacetyl-CoA, (ii) oxidation of phenylacetyl-CoA to phenylglyoxylate, and finally (iii) the oxidative decarboxylation of phenylglyoxylate to benzoyl-CoA. The genes encoding the enzymatic reactions of all three steps are located on the *pad* gene cluster (Fig. 1C). In *S. hydrogenivorans* sk43H, this cluster is located less than 5 kb upstream of the *box* gene cluster. The activation of phenylacetate is probably catalyzed by the gene product of *SUTH_01630*, a CoA ligase (PadJ) (Wöhlbrand *et al.*, 2007). Phenylacetyl-CoA is further oxidized to phenylglyoxylate, presumably by the phenylacetyl-CoA:acceptor oxidoreductase (PadBCD) encoded by *SUTH_01622-01624*. The formation of benzoyl-CoA is probably catalyzed by the gene product of *SUTH_01625-01629,* a phenylglyoxylate:NAD^+ oxidoreductase (PadEFGHI). Benzoyl-CoA is further converted via the upper benzoyl-CoA pathway (see above).

The *pad* gene clusters of *S. hydrogenivorans* sk43H and "*A. aromaticum*" strain EbN1 harbor a second putative CoA ligase encoding gene (*SUTH_01619* and *ebA5403*, respectively). The gene position differs among the clusters (Fig. 1C). This CoA ligase is thought to be specific for the activation of 4-hydroxyphenylacetate to 4-hydroxyphenylacetyl-CoA (Mohamed & Fuchs, 1993; Carmona *et al.*, 2009). It is supposed that PadBCD and PadEFGHI further convert 4-hydroxyphenylacetyl-CoA to 4-

hydroxybenzoyl-CoA as described above. At the 3-prime end of the gene cluster of *S. hydrogenivorans* sk43H (*SUTH_01631*-*SUTH_01634*), a putative tripartite ATP-independent periplasmic (TRAP) transporter is encoded, which might be involved in the uptake of phenylacetate. A similar transporter is present in "*A. aromaticum*" strain EbN1 (*ebA5362*, *ebB188*, *ebA5367*), but not part of the *pad* gene cluster. An aerobic phenylacetate hybrid pathway (*paa* gene cluster) similar to the benzoate hybrid pathway (*box* gene cluster) was not found in *S. hydrogenivorans* sk43H. In "*A. aromaticum*" strain EbN1 two distinct *paa* gene clusters are present (Rabus *et al.*, 2005).

*Anaerobic peripheral pathway of cinnamate conversion (*cou *gene cluster)*

The degradation of phenylpropanoids such as cinnamate and 4-hydroxycinnamate (*p*-coumarate) was studied by proteome analysis in "*A. aromaticum*" strain EbN1. In this organism, a 3-phenylpropanoid degradation cluster (*cou* gene cluster) was identified, which is located directly downstream of the cluster for anaerobic benzoate degradation (Trautwein *et al.*, 2012b). In *S. hydrogenivorans* sk43H, a 3-phenylpropanoid degradation cluster is also present; however, two genes encoding key enzymes are missing (Fig. 1D). The proposed reaction flow for the conversion of cinnamate to benzoyl-CoA is as follows (Trautwein *et al.*, 2012b): (i) activation of cinnamate to cinnamoyl-CoA, (ii) hydration of cinnamoyl-CoA to 3-hydroxy-3-phenylpropanoyl-CoA, (iii) dehydrogenation of the latter to form benzoyl-acetyl-CoA, and finally (iv) the formation of the central intermediate benzoyl-CoA catalyzed by a ketothiolase. In *S. hydrogenivorans* sk43H, the gene product of *SUTH_01231*, a putative 3-phenylpropanoid-CoA ligase, probably catalyzes the activation of cinnamate. Further, the double bond of the side chain of cinnamoyl-CoA might be hydrated by a putative enoyl-hydratase/isomerase (*SUTH_01232*) leading to the formation of 3-hydroxy-3-phenylpropanoyl-CoA. Since the genes encoding the enzymatic reactions of step (iii) and (iv) are absent in the cluster of *S. hydrogenivorans* sk43H, a further conversion of 3-hydroxy-3-phenylpropanoyl-CoA is doubtful. The gene *SUTH_01233*, a homolog of which is not present in "*A. aromaticum*" strain EbN1, possibly encodes a hydrolase. In the soil actinobacterium *Rhodococcus jostii* RHA1, it was shown that a similar hydrolase is essential for the degradation of *p*-hydroxycinnamte by catalyzing the hydrolysis of 4-hydroxy-3-methoxyphenyl-ketopropionate-CoA to vanillate releasing acetyl-CoA (Otani *et al.*, 2014). This enzymatic reaction might replace the reaction of the ketothiolase (step iv) in *S. hydrogenivorans* sk43H. However, the pathway seems to be still interrupted due to the lack of reaction step iii.

The compound hydrocinnamate is also channeled via this pathway (Fig.1D). Its activation to cinnamoyl-CoA is proposed as a two-step reaction (Trautwein *et al.*, 2012b) putatively

involving the gene products of *SUTH_01231* (see above) and *SUTH_01234* (a putative acyl-CoA dehydrogenase). Further components of the *cou* gene cluster are ABC transporter elements (*SUTH_1227-1230*) including a putative periplasmic solute binding protein (*SUTH_01230*), which is possibly involved in the binding of the 3-phenylpropanoids (Giuliani *et al.*, 2011; Trautwein *et al.*, 2012b). The gene of such a binding protein is also present in "*A. aromaticum*" strain EbN1 (*ebA5316*). Homologs of the other ABC transporter elements *SUTH_1227-1229* are absent in strain EbN1.

Lower benzoyl CoA pathway

Aliphatic dicarboxyl-CoA derivatives (e.g. 3-hydroxypimelyl-CoA) are formed as end products of the upper benzoyl CoA pathway. In the lower benzoyl CoA pathway, these metabolites are further degraded to acetyl CoA and CO_2 via β-oxidation (for review see Carmona *et al.*, 2009). In the genome of "*A. aromaticum*" strain EbN1, a variety of genes encoding enzymes putatively involved in β-oxidation were detected (Rabus *et al.*, 2005). For each class of enzyme, several homologs were also identified in *S. hydrogenivorans* sk43H (Tab. S3). The encoding genes are rather scattered in the genome than grouped into clusters.

Aerobic pathways for aromatic compound degradation

Under aerobic conditions, aromatic rings can be attacked by molecular oxygen. Ring hydroxylation and cleavage is catalyzed by hydroxylating oxygenases and ring-cleaving dioxygenases, respectively (for review see Fuchs *et al*., 2011). In *S. hydrogenivorans* sk43H, such enzymes were only identified for the degradation of 4-hydroxybenzoate (*SUTH_01247*: 4-hydroxybenzoate 3-monooxygenase; *SUTH_01241*/*SUTH_01242* + *SUTH_01245*/*SUTH_01246*: protocatechuate 4,5-dioxygenase, two copies). A putative salicylate hydroxylase (*SUTH_02820*), is also encoded in the genome, however, genes encoding enzymes for subsequent oxidative ring cleavage were not found.

Cultivation of S. hydrogenivorans *sk43H with aromatic compounds*

Twenty aromatic compounds (naturally abundant or crude-oil-derived) were tested as growth substrate for *S. hydrogenivorans* sk43H applying nitrate-reducing conditions. We focused on aromatic carboxylic acids and their *para*-hydroxylated or -alkylated derivatives. Benzoate (1 mM), which was already reported as growth substrate, was degraded within 20 days coupled to nitrate reduction (Fig. 2A). Nitrite was not detected as an intermediate. The end product of nitrate reduction was probably dinitrogen (Kojima & Fukui, 2011). Further, degradation of 4-hydroxybenzoate, phenylacetate, and 4-hydroxyphenylacetate was observed (Fig. 2B-D). Degradation of phenylacetate was fastest and occurred within 6

days. The hydroxylated aromatics were consumed within 25 days. During the degradation of phenylacetate and 4-hydroxyphenylacetate, phenylglyoxylate (50 µM) and 4-hydroxyphenylglyoxylate (350 µM) accumulated, respectively. The formation of these products is in accordance with the proposed degradation pathway (Fig. 1C). Transient accumulations of (4-hydroxy)phenylglyoxylate during (4-hydroxy)phenylacetate degradation were reported before (Seyfried *et al.*, 1991; Mohamed *et al*., 1993), however, an accumulation of these intermediates as end products is unusual. An explanation may be found in the inhibition of the phenylglyoxylate:acceptor oxidoreductase, which catalyzes the conversion of (4-hydroxy)phenylglyoxylate to (4-hydroxy)benzoyl-CoA. This enzyme was described to be oxygen-sensitive in other organisms (Hirsch *et al.*, 1998) and might have been inhibited by residual amounts of oxygen during cultivation.

No degradation was observed for anthranilate, 4-aminobenzoate, cinnamate, *p*-cresol, 4-ethylbenzoate, 4-hydroxycinnamate, 4-isopropylbenzoate, 2-methylbenzoate, 3-methylbenzoate, 4-methylbenzoate, 4-methylcinnamate, 4-methylphenylacetate, nicotinate, phenylalanine, toluene, and *p*-xylene (Fig. 2F).

Discussion

In this study, we identified several clusters for anaerobic aromatic compound degradation in the genome of *Sulfuritalea hydrogenivorans* sk43H and proved its functionality by cultivation. Simple aromatic carboxylic acids served as growth substrates for S. *hydrogenivorans* sk43H. These compounds are abundant in nature and mainly produced by plants (Umezawa *et al.*, 2010; Sugawara *et al.*, 2015; Widhalm *et al.*, 2015) or formed during the decomposition of plant litter (for review see Bugg *et al.*, 2011). It is likely that the monoaromatics utilized by *S. hydrogenivorans* sk43H were also available in its original habitat, an artificial freshwater lake in Japan situated in a rural mountain area (Kojima *et al*., 2009 & 2011). Industrially produced aromatic compounds such as toluene and *p*-cresol were not degraded by *S. hydrogenivorans* sk43H. The frequent occurrence of *Sulfuritalea* spp. at industrial polluted sites (Juretschko *et al.*, 2002; Yagi *et al.*, 2010; Martin *et al.*, 2012; Guo *et al.*, 2017; Keller *et al.*, 2017; Sperfeld *et al.*, 2018) might be explained by the utilization of aromatic carboxylic acids such as 4-hydroxbenzoate, which might be released by other anaerobic bacteria during the degradation of e.g. *p*-cresol or phenol (Bossert *et al.*, 1986; Rudolphi *et al.*, 1991). If *Sulfuritalea*-related microorganisms are also able to utilize industrially produced aromatics, cannot be excluded and needs to be further elucidated.

The gene clusters for aromatic compound degradation that were identified in *S. hydrogenivorans* sk43H were similar to clusters present in the genome of "*A. aromaticum*" strain EbN1. Strain EbN1 was affiliated to the genus *Azoarcus*, which comprises two

ecophysiological groups: (i) free-living *Azoarcus* that anaerobically degrade a large number of aromatic compounds (Rabus and Widdel, 1995; Song *et al.*, 1999; Lee *et al.*, 2014) and (ii) endophytes that invade plant roots (Reinhold-Burek *et al.*, 1993; Hurek *et al.*, 1997). The genomes of the free-living strain EbN1 and of the facultative endophyte *Azoarcus* sp. strain CIB are both characterized by the presence of an extensive mobilome, including high numbers of transposases, phage-related integrases/recombinases, and genomic islands that were most probably acquired during horizontal gene transfer (Rabus *et al.*, 2005; Martin-Moldes *et al.*, 2015). It was suggested that these mobile elements allow *Azoarcus* spp. to adapt to free-living and/or endophytic conditions (Martin-Moldes *et al.*, 2015). Considering that 5% of the genes of *S. hydrogenivorans* sk43H have highest similarity to genes of "*A. aromaticum*" strain EbN1 (Table S2 in Watanabe *et al.*, 2014), it seems feasible that members of *Azoarcus* and *Sulfuritalea* exchange genetic information among each other. The exchange of genetic information requires that both genera co-occur at the same habitat, which was shown e.g. for a BTEX-polluted aquifer (Sperfeld *et al.*, 2018).

In the genome of *S. hydrogenivorans* sk43H, all functional gene clusters for anaerobic aromatic compound degradation including the *box* gene cluster are located in close proximity forming a ~50 kb-gene region. Benzoate, 4-hydroxybenzoate, phenylacetate, and 4-hydroxyphenylacetate were consumed. The utilization of 4-hydroxybenzoate was not expected since the requirement of an additional benzoyl-CoA reductase for the conversion of this compound was reported for *Thauera aromatica* (strain K172) (Brackmann & Fuchs, 1993). This molybdenum-containing 4-hydroxybenzoyl-CoA reductase, which is not encoded in the genome of *S. hydrogenivorans* sk43H, removes the *para*-positioned hydroxyl group of 4-hydroxybenzoyl-CoA prior to dearomatization of the ring (Brackmann & Fuchs, 1993). In *S. hydrogenivorans* sk43H, only a single class I benzoyl-CoA reductase is encoded. This allows two suggestions for the reaction mechanism: either the hydroxyl group of 4-hydroxybenzoyl-CoA is retained during the dearomatization step or the class I benzoyl-CoA reductase of *S. hydrogenivorans* sk43H has an intrinsic activity to remove the *para*-positioned hydroxyl group prior to dearomatization. This has to be clarified in future studies. A strict dependence on a specific 4-hydroxybenzoyl-CoA reductase for the degradation of 4-hydroxybenzoate was also questioned in "*A. aromaticum*" strain EbN1. In this organism, a 4-hydroxybenzoyl-CoA reductase is encoded, but the gene product was not found in the proteome when cultivated with 4-hydroxybenzoate (Wöhlbrand *et al.*, 2007).

It should be noted that the degradation rate of benzoate for *S. hydrogenivorans* sk43H is about 20fold lower compared to well-adapted anaerobic aromatic compound degraders such as *Azoarcus* spp. and *Thauera* spp. (Philipp and Schink, 2000; Trautwein *et al.*, 2012a). However, *Sulfuritalea*-related species were observed to be enriched with *p*-xylene

as electron donor (Rotaru *et al.*, 2010; originally assigned as *Denitratisoma*-related, but higher 16S rRNA nucleotide identity to *Sulfuritalea*), and outcompeted *Azoarcus* spp. in enrichment cultures amended with *para*-alkylated aromatic compounds (Sperfeld *et al.*, unpublished). In conclusion, it is feasible that *Sulfuritalea* spp. are not only important chemolithoautotrophic sulfur oxidizers, but also play an active role in the anaerobic degradation of aromatic compounds in the environment.

Figure 1: Proposed pathways for anaerobic aromatic compound degradation in *Sulfuritalea hydrogenivorans* sk43H (GenBank No. AP012547.1) and organization of the gene clusters in comparison to „*Aromatoleum aromaticum*“ strain EbN1 (GenBank No. CR555306.1). (**A**) Anaerobic degradation of benzoate and the upper benzoyl CoA pathway (*bcr/bzd* gene cluster). (**B**) Aerobic benzoate hybrid pathway (*box* gene cluster). (**C**) Anaerobic peripheral pathway of phenylacetate (*pad* gene cluster). (**D**) Anaerobic peripheral pathway of cinnamate (*cou* gene cluster). The nucleotide sequence identity of related genes of *S. hydrogenivorans* sk43H and „*A. aromatoleum*“ strain EbN1 involved in aromatic compound degradation as well as a functional description of the gene products is given in Table S2. The *box* gene cluster of *S. hydrogenivorans* sk43H, which is marked with an asterisk, is located on the reverse strand and shown in inverted orientation. The figure was adapted from Rabus *et al*. (2005), Carmona *et al*. (2009), Rather *et al*. (2010) and Trautwein *et al*. (2012b).

Figure 2: Degradation of (**A**) (1) benzoate, (**B**) (2) 4-hydroxybenzoate, (**C**) (3) phenylacetate, and (**D**) (5) 4-hydroxyphenylacteate by *Sulfuritalea hydrogenivorans* sk43H under nitrate-reducing conditions. The intermediates formed during cultivation with phenylacetate and 4-hydroxyphenylaceate were (4) phenylglyoxylate and (6) 4-hydroxyphenylglyoxylate, respectively. The filled circle (●) indicates the aromatic substrate, the open circle (○) indicates the intermediate formed, and the open triangle (△) shows nitrate. (**E**) Bright-field microphotograph of *S. hydrogenivorans* sk43H cells grown with benzoate and nitrate. (**F**) No substrate utilization was observed for (7) 4-methylbenzoate, (8) 3-methylbenzoate, (9) 2-methylbenzoate, (10) 4-ethylbenzoate, (11) 4-isopropylbenzoate, (12) nicotinate, (13) anthranilate, (14) 4-aminobenzoate, (15) 4-methylphenylacetate, (16) phenylalanine, (17) cinnamate, (18) 4-hydroxycinnamate, (19) 4-methylcinnamate, (20) *p*-cresol, (21) toluene, and (22) *p*-xylene.

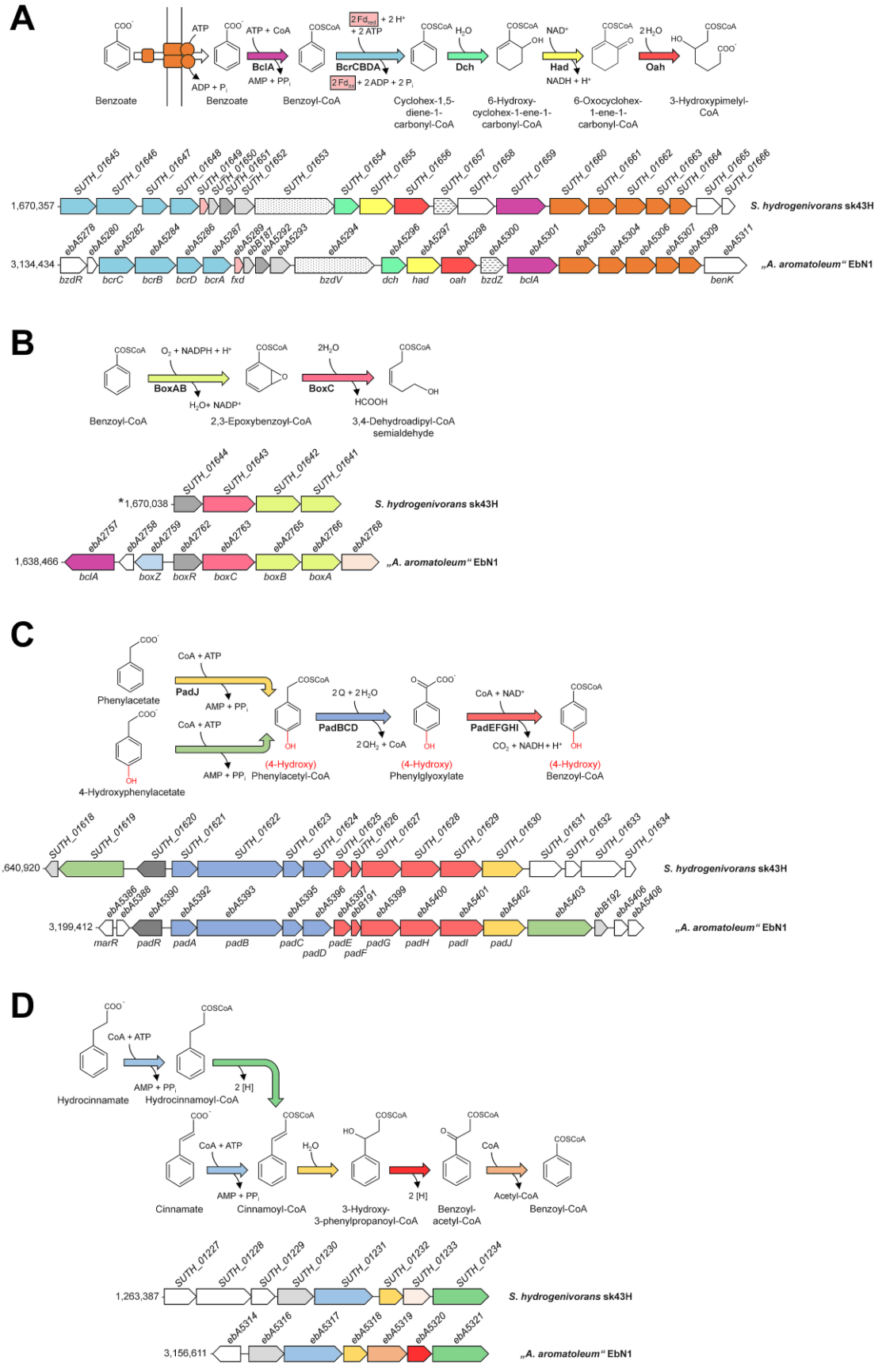

A
Benzoate
ATP
ADP + Pi
Benzoate
ATP + CoA
AMP + PPi
BclA
Benzoyl-CoA
2 Fdred + 2 H+ + 2 ATP
2 Fdox + 2 ADP + 2 Pi
BcrCBDA
Cyclohex-1,5-diene-1-carbonyl-CoA
H2O
Dch
6-Hydroxy-cyclohex-1-ene-1-carbonyl-CoA
NAD+
NADH + H+
Had
6-Oxocyclohex-1-ene-1-carbonyl-CoA
2 H2O
Oah
3-Hydroxypimelyl-CoA
1,670,357
S. hydrogenivorans sk43H
3,134,434
„A. aromatoleum" EbN1
bzdR
bcrC
bcrB
bcrD
bcrA
fxd
bzdV
dch
had
oah
bzdZ
bclA
benK
B
Benzoyl-CoA
O2 + NADPH + H+
BoxAB
H2O+ NADP+
2,3-Epoxybenzoyl-CoA
2H2O
BoxC
HCOOH
3,4-Dehydroadipyl-CoA semialdehyde
*1,670,038
S. hydrogenivorans sk43H
1,638,466
„A. aromatoleum" EbN1
bclA
boxZ
boxR
boxC
boxB
boxA
C
Phenylacetate
4-Hydroxyphenylacetate
CoA + ATP
PadJ
AMP + PPi
(4-Hydroxy) Phenylacetyl-CoA
2 Q + 2 H2O
PadBCD
2 QH2 + CoA
(4-Hydroxy) Phenylglyoxylate
CoA + NAD+
PadEFGHI
CO2 + NADH + H+
(4-Hydroxy) Benzoyl-CoA
1,640,920
S. hydrogenivorans sk43H
3,199,412
„A. aromatoleum" EbN1
marR
padR
padA
padB
padC
padD
padE
padF
padG
padH
padI
padJ
D
Hydrocinnamate
CoA + ATP
AMP + PPi
Hydrocinnamoyl-CoA
2 [H]
Cinnamate
CoA + ATP
AMP + PPi
Cinnamoyl-CoA
H2O
3-Hydroxy-3-phenylpropanoyl-CoA
2 [H]
Benzoyl-acetyl-CoA
CoA
Acetyl-CoA
Benzoyl-CoA
1,263,387
S. hydrogenivorans sk43H
3,156,611
„A. aromatoleum" EbN1

Figure 1

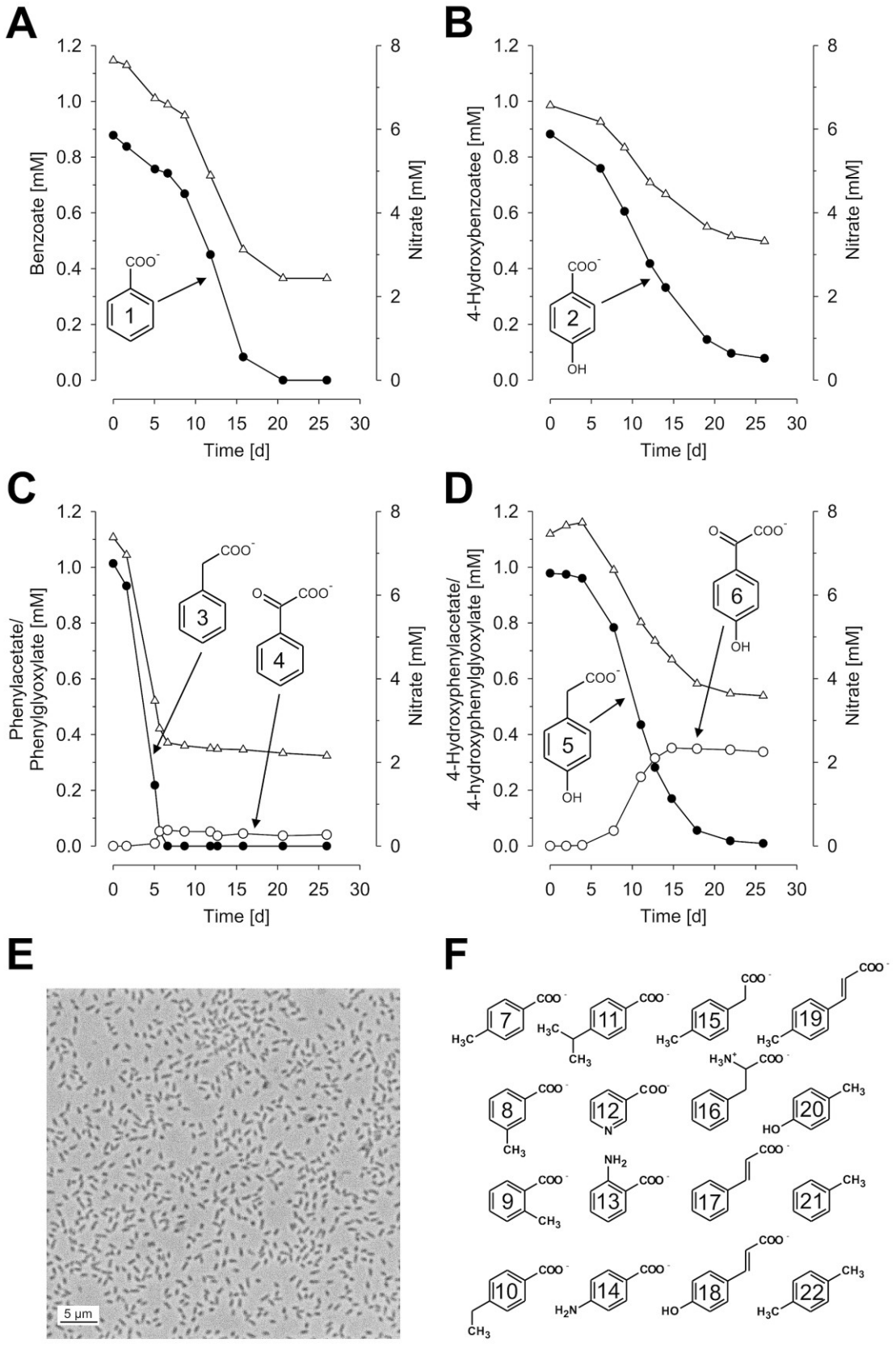

A
Benzoate [mM]
Nitrate [mM]
Time [d]
B
4-Hydroxybenzoatee [mM]
Nitrate [mM]
Time [d]
C
Phenylacetate/
Phenylglyoxylate [mM]
Nitrate [mM]
Time [d]
D
4-Hydroxyphenylacetate/
4-hydroxyphenylglyoxylate [mM]
Nitrate [mM]
Time [d]
E
5 µm
F

Figure 2

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2.4 MANUSCRIPT III

Title: Community dynamics in a nitrate-reducing microbial consortium cultivated with *p*-alkylated vs. non-*p*-alkylated aromatic compounds

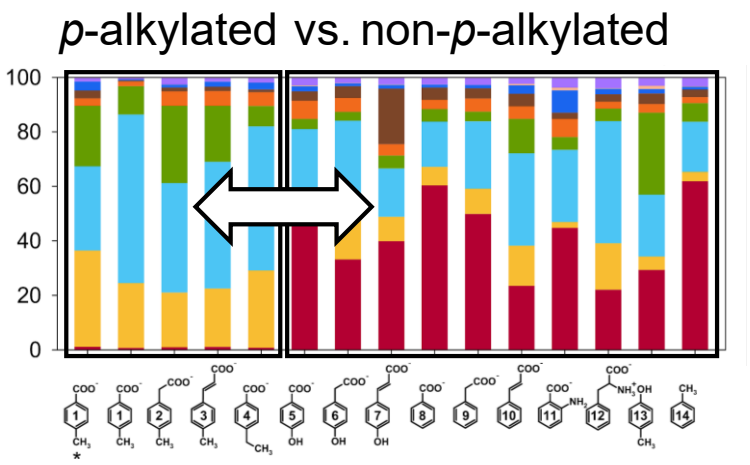
Authors: Sperfeld M, Diekert G, Studenik S

Current state: Submitted to FEMS Microbiology Ecology, date of submission: 24/May/2018

Supplementary materials: see chapter V, p. xvii - xxi

Abstract:

In this study, we established the nitrate-reducing, aromatic-compound degrading enrichment culture pMB18. Its community structure was controlled by the aromatic substrate applied: In the presence of a *p*-alkylated substrate, microorganisms related to *Sulfuritalea*, *Comamonadaceae* and *Ignavibacterium* were abundant. Non-*p*-alkylated structural analogues promoted the enrichment of *Azoarcus* probably favored by the excretion of nitrite. The analysis of the *bam* gene, which is a functional marker for anaerobic aromatic compound degradation, as wells as a differential abundance analysis suggested the involvement of *Sulfuritalea* and *Comamonadaceae* in the degradation of *p*-alkylated substrates. Members of the genus *Azoarcus* were assumed to be the key players for the degradation of the non-*p*-alkylated substrates. A gene cluster encoding a putative 4-methylbenzoyl-CoA reductase, which is supposed to be specific for the dearomatization of *p*-alkylated benzoyl-CoA intermediates, was detected in culture pMB18 dominated by *Sulfuritalea*, *Ignavibacterium*, and *Comamonadaceae*, but not in an *Azoarcus*-dominated culture. This study allowed insight into a microbial community, whose composition was guided by the aromatic substrate applied.



Introduction

Aromatic ring structures show a high chemical stability due to the circulating π electrons within the aromatic ring system (for review see Fuchs *et al.*, 2011). Consequently, aromatic compounds are ideal building blocks in living material (e.g. monolignols, aromatic amino acids); however, they also tend to accumulate under anoxic conditions. In recent years, tremendous advances were made in the understanding of the reactions, mechanisms and microorganisms involved in anaerobic aromatic compound degradation (see special issue *J Mol Microbiol Biotechnol* 2016, Vol. 26, No. 1-3 'Anaerobic biodegradation of hydrocarbons'; synopsis by Rabus *et al*., 2016b). The first step of anaerobic aromatic compound degradation comprises a set of peripheral pathways that channel a large variety of aromatic compounds into a limited number of central intermediates (e.g. benzoyl-CoA). In this process, substituents attached to the aromatic ring are mostly removed; if retained benzoyl-CoA analogues with an additional hydroxyl, alkyl, amino or halide group are formed (for reviews see Carmona *et al.*, 2009; Boll *et al.*, 2014). In the following, the energy-demanding dearomatization of the resonance-stabilized aromatic ring takes places, which is catalyzed by class I or class II benzoyl-CoA reductases (BCR) (for review see Boll 2005; Kung *et al.* 2010). Previous studies on class I BCR point to mechanistic challenges that will arise from an alkyl substituent attached in *para*-position to the CoA thioester group of the aromatic ring (Möbitz and Boll, 2002; for reviews see Buckel *et al.*, 2014; Rabus *et al.*, 2016a): It is proposed that after an initial single electron transfer to the aromatic ring, which results in the formation of a radical-anion intermediate, the *para*-position gets protonated (Buckel *et al.*, 2014). An alkyl substituent in this position may hinder this proton transfer. In addition, the *p*-alkyl group might destabilize the radical-anion intermediate. To overcome these restrictions, a specific 4-methylbenzoyl-CoA reductase (4-MBCR) seems to be required, which was identified in *Magnetospirillum* sp. pMbN1 for the degradation of *p*-methylbenzoate (Lahme *et al.*, 2012). A gene cluster putatively encoding a 4-MBCR was also detected in a *p*-xylene-degrading enrichment culture originating from a gasworks site (Sperfeld *et al.*, 2018).

The ability of microorganisms to anaerobically degrade *para*-alkylated aromatic compounds differs among investigated sites (Widdel *et al.*, 2010; Lahme *et al.*, 2012; Rabus *et al.*, 2016a). In earlier studies, *para*-alkylated toluenes such as *p*-xylene were observed to be particularly recalcitrant (Rabus *et al.*, 1996 & 1999; Wilkes *et al.*, 2000). It was further noticed that the degradation may stop with the formation of the respective *para*-alkylated benzoate as dead end product (Biegert and Fuchs, 1995; Rabus and Widdel, 1995; Beller, 1996). This is reasonable considering the above mentioned challenges to dearomatize *para*-alkylated ring systems. Nevertheless, anaerobic *p*-xylene- or *p*-methylbenzoate-

degrading enrichment cultures were successfully established from samples of various freshwater ecosystems (Häner *et al.*, 1995; Wu *et al.*, 2001; Morasch and Meckenstock, 2005; Rotaru *et al.*, 2010). In enrichment cultures originating from a gasworks aquifer in Thuringia, the degradation of *p*-xylene was even faster than the degradation of *m*- and *o*-xylene (Sperfeld *et al.*, 2018). Only a few pure cultures exist that degrade *p*-alkylated benzoates or toluenes with either nitrate or sulfate as electron acceptor (Higashioka *et al.*, 2012; Lahme *et al.*, 2012; Strijkstra *et al.*, 2014). Microorganisms involved in the degradation of *para*-alkylated aromatic compounds were affiliated to the genera "*Aromatoleum*", *Azoarcus*, *Thauera*, *Sulfuritalea* (originally assigned as *Denitratisoma*-related), *Georgfuchsia* (all Betaproteobacteria), *Magnetospirillum* (Alphaproteobacteria), and *Desulfosarcina* (Deltaproteobacteria) (Morasch and Meckenstock, 2005; Rotaru *et al.*, 2010; Higashioka *et al.*, 2012; Lahme *et al.*, 2012; Strijkstra *et al.* 2014; Sperfeld *et al.*, 2018).

To extend the knowledge about microorganisms involved in the degradation of *p*-alkylated aromatic compounds, we further characterized the microbial communities originating from the Thuringia gasworks site (Sperfeld *et al.*, 2018). By changing the alkylated substrate from *p*-xylene to *p*-methylbenzoate, the enrichment culture pMB18 was established. This culture undergoes specific shifts in the composition of the microbial community in response to *p*-alkylated and non-*p*-alkylated aromatic growth substrates. The results of cultivation, 16S rRNA-, and *bamA*-analysis allowed insight into the microorganisms involved in the degradation of *p*-alkylated aromatics.

Materials and Methods

Microbial cultivation

For cultivation experiments, a mineral salt medium was used described in Sperfeld *et al.* (2018) with slight modifications: the vitamin stock solution further contained vitamin B_{12}, riboflavin, folic acid, and α-lipoic acid (10 mg/l each). To obtain culture pMB18, the *p*-xylene-degrading enrichment culture described in Sperfeld *et al.* (2018) was adapted to *p*-methylbenzoate degradation and then subjected to a single anoxic isolation step on agar. Anoxic agar was prepared in 150 ml-glass flasks. Therefore, 1.2 % (w/v) agar was added to 30 ml mineral salt medium. The gas atmosphere was changed to 100% nitrogen. After autoclaving, the temperature of the medium was adjusted to 55°C and amended with the following supplements: 30 mM $NaHCO_3$, 150 µl vitamin stock solution, 30 µl trace element stock solution, 10 mM $NaNO_3$ and 1 mM *p*-methylbenzoate. After addition of diluted culture samples (500 µl) and cautious mixing, the agar was solidified on ice. The agar-containing flasks were incubated in the dark at 24°C for four weeks. Single colonies were picked under anoxic conditions by inserting a Pasteur pipette into agar and transferring the colonies to

10 ml of fresh mineral salt medium containing 10 mM $NaNO_3$ and 1 mM *p*-methylbenzoate. The resulting culture pMB18 was cultivated in anoxic 200 ml-serum flasks filled with 100 ml mineral salt medium containing 7.5 mM nitrate and 1 mM *p*-methylbenzoate. The culture was regularly transferred using 10% (v/v) inoculum. To analyze the substrate utilization pattern of culture pMB18, stock solutions of the following compounds were prepared in ultrapure water (25 mM each, pH 7, adjusted with NaOH): anthranilate, *p*-aminobenzoate, benzoate, cinnamate, *p*-ethylbenzoate, *p*-hydroxybenzoate, *p*-hydroxycinnamate, *p*-hydroxyphenylacetate, *p*-isopropylbenzoate, *o*-methylbenzoate, *m*-methylbenzoate, *p*-methylbenzoate, *p*-methylcinnamate, *p*-methylphenylacetate, nicotinate, phenylacetate, phenylalanine and *p*-cresol. For toluene and *p*-xylene, stock solutions (250 mM each) were prepared in 2,2,4,4,6,8,8-heptamethylnonane (HMN). Cultivation took place in anoxic 50 ml-serum bottles filled with 25 ml mineral salt medium containing 7.5 mM nitrate and 25 µmol of the aromatic substrate. As inoculum, 1 ml of culture pMB18 grown with nitrate and *p*-methylbenzoate was applied. Incubation of cultures was done in the dark at 24°C.

Analytical methods

Nitrate, nitrite and polar aromatic compounds were quantified using a high performance liquid chromatography system (Merck-Hitachi) equipped with a LiChrospher® 100 RP-18 (5 mm) LiChroCART® 125-4 column (Merck KGaA, Darmstadt, Germany). A flow rate of 0.4 ml min^{-1} was applied and signals were detected at 220 nm. Methanol in 0.3% (v/v) H_3PO_4 was used as eluent applying the following gradient: 5% (v/v) methanol (0 - 2 min), increase to 70% (v/v) methanol (2 - 9 min), hold 70% (v/v) methanol (9 - 19 min), decrease to 5% (v/v) methanol (19 - 20 min), hold 5% (v/v) methanol (20 - 40 min). Retention times of analytical standards were as follows: nitrate (3.2 min), nicotinate (4.7 min), *p*-aminobenzoate (5.9 min), nitrite (7.2 min), *p*-hydroxyphenylglyoxylate (8.5 min), *p*-hydroxybenzyl alcohol (9.5 min), anthranilate (10.4 min), phenylalanine (10.5 min), *p*-hydroxyphenylacetate (13.6 min), *p*-hydroxybenzoate (14.0 min), *p*-methylphenylglyoxylate (15.2 min), *p*-hydroxycinnamate (15.4 min), phenylacetate (15.8 min), *p*-cresol (16.4 min), benzoate (16.7 min), *o*-methylbenzoate (17.2 min), *m*-methylbenzoate (17.6 min), cinnamate (17.7 min), *p*-methylphenylacetate (17.7 min), *p*-methylbenzoate (18.4 min), p-ethylbenzoate (19.6 min), *p*-methylcinnamate (21.1 min), and *p*-isopropylbenzoate (22.0 min). The formation of benzoate, *p*-hydroxybenzoate, *p*-methylbenzoate, *p*-hydroxybenzyl alcohol, *p*-hydroxyphenylglyoxylate, and *p*-methylphenylglyoxylate as intermediates during cultivation was confirmed by addition of analytical standards to the respective culture samples. An unknown intermediate (HPLC retention time 16.3 min) formed during the cultivation with *p*-methylcinnamate was identified as 3-hydroxy-3-(4-methylphenyl)propanate by high resolution LC/MS analysis. Since no standard for HPLC

was available, the concentration of 3-hydroxy-3-(4-methylphenyl)propanate was estimated using the calibration curve of *p*-methylcinnamate. Toluene and *p*-xylene were analyzed by headspace gas chromatography according to Sperfeld *et al.* (2018).

16S rRNA Illumina-sequencing

Samples of culture pMB18 (10 ml) were taken at the late exponential growth phase and centrifuged for 45 min (10,000 x g, 4°C). The DNA was isolated using the innuPREP Bacteria DNA kit according to the manufacturer's protocol (Analytik Jena AG, Jena, Germany). DNA quantification was done using a Qubit® fluorometer and the Qubit® dsDNA BR assay kit (Thermo Fisher Scientific GmbH, Dreieich, Germany). The mean DNA concentration was 18 ng/µl (± 9 SD). The DNA was used for 16S rRNA paired-end sequencing on an Illumina MiSeq system, performed by a sequencing company (MR DNA®, Shallowater, TX, USA). The primers S-D-Bact-0341-b-S-17 (5'-CCT ACG GGN GGC WGC AG-3') and S-D-Bact-0785-a-A-21 (5'-GAC TAC HVG GGT ATC TAA TCC-3') were used to target the V3/V4 region of bacterial 16S rRNA (Klindworth *et al.*, 2012). Barcodes were attached to the forward primer. Illumina raw sequences were analyzed using QIIME 2 (version 2018.2; https://qiime2.org; Caporaso *et al.*, 2010). The applied QIIME 2 scripts are given in the supplement (Qiime_Scripts.docx). The following workflow was used: (i) joining of paired-end sequences, (ii) PHRED-based filtering, (iii) trimming of forward and reverse primers, (iv) quality filtering with DADA2, and (v) taxonomic assignment using a pre-trained QIIME 2 compatible SILVA database (release 128, 97% clustering identity, 90% majority taxonomy strings) (Aronesty, 2011; Bokulich *et al.*, 2013 & 2018; Callahan *et al.*, 2016; Martin, 2011; Quast *et al.*, 2013). After processing, an average of 25,479 (± 4,350 SD) sequences was detected per sample. They consisted of 67 unique 16S rRNA sequences with a mean length of 417 bp (± 15 SD). The unique sequences were collapsed at the genus level into 38 operational taxonomic units (OTUs). The outcome of the QIIME 2 analysis is given as supplemental table (Tab. S1). This table summarizes the performance of sequence filtering, 16S rRNA nucleotide sequences, relative abundances within samples, taxonomic assignment, and OTU affiliations. To investigate substrate-dependent community composition changes, a differential abundance analysis was performed using ANCOM implemented in QIIME 2 (Mandal *et al.*, 2015; Weiss *et al.*, 2017). ANCOM counts, how often the log-ratio of abundance of a single OTU significantly differs to all remaining OTUs one at a time (*W*). These *W*-values were plotted against logarithmized and centered differences in mean relative abundances (*clr*).

Preparation of bamA *clone libraries*

The analysis of *bamA* gene diversity was conducted as described before (Sperfeld *et al.*, 2018). Briefly, the *bamA* gene was PCR-amplified using the oah_f (5'-GCA GTA CAA YTC CTA CAC SAC YGA BAT GGT-3') and oah_r (5'-CCR TGC TTS GGR CCV GCC TGV CCG AA-3') primers published by Staats *et al.*, 2011. As template, the same DNA was used as for 16S rRNA analysis. The TOPO® TA cloning® kit (Thermo Fisher Scientific GmbH, Dreieich, Germany) was used for library preparation. Plasmids were isolated from positive transformants and sent to GATC Biotech AG (Konstanz, Germany) for Sanger sequencing. Sequences were analyzed with the MEGA 7 software (Kumar *et al.*, 2016). A phylogenetic tree was inferred by the maximum likelihood method and 1,000 bootstrap replicates (Nei and Kumar, 2000). Sequence data are summarized in Table S2.

Detection of a putative 4-methylbenzoyl-CoA reductase-encoding gene cluster

For detection of the 4-MBCR gene cluster, the degenerated primers mbrc317_FW (5'-TGT TYG TYA CCC AYC CKA TCT G-3') and mbrc1031_RV (5'-AGA CCB GGY TCR CAC ATC TTG-3') were used as previously described (Sperfeld *et al.*, 2018). For sequencing of the almost complete 4-MBCR gene cluster (~4 kb), two additional fragments were PCR-amplified. Fragment 1 was obtained by using the primers MBCR_0021_FW (5'-ATG GGC CTG ATA CC-3') and MBCR_1513_RV (5'-CCG CTC ATA CTC ATT GCG TAG-3'). Fragment 2 was obtained with the primers MBCR_1888_FW (5'-GCT GTT GGA AGA TGT GAG CAC-3') and MBCR_4136_RV (5'-GGA AAG CGT TGT TAT TCG -3').

Fluorescence in situ *hybridization (FISH)*

For the visual detection of *Sulfuritalea*-related microorganisms by FISH, the Cy3-labeled probe STA442 (5'-CAC CGT TTC GTT CCT GC-3') described in Watanabe *et al.* (2017) was used. Hybridization of cells was performed on glass slides according to Manz *et al.* (1992). The buffer for hybridization contained 900 mM NaCl, 20% (v/v) formamide, 0.01% (w/v) SDS and 20 mM Tris HCl pH 8 as well as 5 ng of the probe. Hybridization was performed at 42°C for 2 h. The washing buffer contained 225 mM NaCl, 0.01% (w/v) SDS and 20 mM Tris HCl pH 8. Washing was performed at 48°C for 20 min. Subsequently, bacterial DNA was stained by addition of 4'-6-diamidino-2-phenylindole (DAPI; 1 ng/µl). After rinsing with ultrapure water and drying, one drop of SlowFade antifade reagent (Thermo Fisher Scientific, Darmstadt, Germany) was added to each well and covered with a cover slip. Epifluorescence images were taken with an AxioCam MRm equipped to an Axio Scope A1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

Results

Establishment of the enrichment culture pMB18

The culture pMB18 originates from a *p*-xylene-degrading enrichment culture, which was previously obtained from a gasworks aquifer in Thuringia (Germany) described in Sperfeld *et al.* (2018). This *p*-xylene-degrading culture was dominated by members of the genus *Georgfuchsia* (Sperfeld *et al.*, 2018). Here, we attempted to identify and isolate key players from this culture, which are involved in the degradation of *p*-alkylated aromatic compounds. The initial electron donor *p*-xylene was replaced by *p*-methylbenzoate, which has a higher solubility in water and is less toxic. Nitrate was used as electron acceptor. After adaption of the initial *p*-xylene-degrading enrichment culture to *p*-methylbenzoate, we applied a single anoxic isolation step on agar followed by several transfers on fresh liquid medium containing *p*-methylbenzoate. This procedure resulted in the establishment of the culture pMB18, which showed a homogenous morphology in microscopic observation (Fig. 1). Community analysis revealed that the culture was dominated by three different OTUs, which were affiliated to *Sulfuritalea, Ignavibacterium,* and *Comamonadacae*, respectively (Fig. 2A, marked with an asterisk). The three OTUs were evenly distributed in culture pMB18 (relative abundances of 35%, 31%, and 22%, respectively). The relative abundance of *Sulfuritalea*-related microorganisms was confirmed using FISH (Fig. S1). No 16S rRNA sequences were affiliated to *Georgfuchsia toluolica* G5G6. This indicates that this microorganism, most dominant in the *p*-xylene enrichment culture of the gasworks site, was lost in the culture pMB18 as a result of the isolation step on agar. The maximum rate of *p*-methylbenzoate conversion was 0.2 mM per day (Fig. S2, substrate 1), which is roughly ten times slower than observed for *Magnetospirillum* sp. pMbN1 (Lahme *et al.*, 2012). Similar to *Magnetospirillum* sp. pMbN1, no intermediate formation of nitrite was observed. The consumption of 1 mol *p*-methylbenzoate was coupled to the conversion of up to 6 mol of nitrate. This suggests a complete denitrification of nitrate to N_2.

Substrate utilization pattern of culture pMB18

Various aromatic compounds were tested as possible growth substrates for culture pMB18. These compounds were classified into two groups: Group A comprised monoaromatics that have an alkyl group in *para*-position to the carboxyl substituent. Substrates within this group were expected to require a specific 4-methylbenzoyl-CoA reductase (4-MBCR) for the dearomatization of the ring structure. Group B included structural analogs of group A (e.g. *p*-hydroxylation instead of *p*-alkylation) as well as naturally abundant or crude oil-derived model compounds. For group B, a specific 4-MBCR should not be required. To determine the substrate utilization pattern, culture pMB18 adapted to *p*-methylbenzoate was used as

inoculum. Within group A, *p*-methylbenzoate, *p*-methylphenylacetate, *p*-methylcinnamate and *p*-ethylbenzoate served as growth substrates (Tab. 1). No growth was observed with *p*-isopropylbenzoate or *p*-xylene. Within group B, anthranilate, benzoate, cinnamate, *p*-cresol, *p*-hydroxybenzoate, *p*-hydroxyphenylacetate, *p*-hydroxycinnamate, phenylacetate, and phenylalanine supported growth. No substrate conversion occurred in the presence of *m*-methylbenzoate, *o*-methylbenzoate, *p*-aminobenzoate or nicotinate. Substrate conversion rates as well as identified intermediates are summarized in Table 1. Substrate conversion curves are depicted in Figure S2. The cultures differed in respect to the formation of nitrite: in case of the *para*-alkylated aromatic compounds of group A, no nitrite was detected, whereas all compounds of group B, if consumed, led to the formation of nitrite. Microscopic observations revealed that a new morphotype occurred during cultivation with compounds of group B (Fig. 1; exemplified for benzoate, phenylacetate and its *p*-hydroxylated analogs).

Community composition analyzed by deep 16S rRNA sequencing

Possible changes of the microbial community in response to different aromatic growth substrates were analyzed by 16S rRNA amplicon sequencing (Illumina MiSeq) of the V3/V4 region. The cultivation of culture pMB18 with *p*-alkylated aromatic compounds different from *p*-methylbenzoate had no obvious influence on the microbial community composition (Fig. 2A, group A). The cultures are still dominated by the OTUs affiliated to *Sulfuritalea, Ignavibacterium,* and *Comamonadacae*, When the culture pMB18 was transferred to medium containing non-*p*-alkylated aromatic compounds of group B, an additional OTU affiliated to the genus *Azoarcus* became abundant (up to 62%; Fig. 2A, group B).

To investigate the substrate-dependent changes of the community composition in more detail, the ANCOM procedure was used (Analysis of composition of microbiomes; Mandal *et al.*, 2015; Weiss *et al.*, 2017). This statistical procedure allows to compare the relative abundances between two or more populations. Here, the procedure was used to identify OTUs that were differentially abundant in culture pMB18 after cultivation with either group A or group B compounds. ANCOM counts, how often the log-ratio of the abundance of a single OTU significantly differs to all remaining OTUs one at a time (*W*). These *W*-values were plotted against logarithmized and centered differences in mean relative abundances (*clr*) (Fig. 2B). As expected, the *Azoarcus*-OTU was significantly higher abundant ($W = 36$) upon cultivation with the non-*p*-alkylated compounds of group B. The *Sulfuritalea*-OTU ($W = 10$) and the *Comamonadaceae*-OTU ($W = 8$) were higher abundant when group A compounds were used as substrates. The abundance of the *Ignavibacterium*-OTU seemed to be less influenced by the supplied aromatic growth substrate ($W = 4$).

Community composition analyzed by bamA *clone library sequencing*

Changes in the microbial community were further analyzed by *bamA* clone library sequencing. The *bamA* gene is a functional marker for anaerobic aromatic compound degradation and encodes a ring-opening hydrolase (Staats *et al*., 2011). For *bamA* clone-library sequencing, the same DNA samples were used as for deep 16S rRNA sequencing. The *bamA* gene diversity was analyzed in culture pMB18 grown with either *p*-methylbenzoate or benzoate. Sequences were clustered into *bamA* OTUs based on the nucleotide identity to *bamA* reference sequences of bacterial isolates (Fig. 3, Tab. S2). The 23 clones analyzed for the *p*-methylbenzoate-grown culture pMB18 were clustered into three *bamA* OTUs. The *bamA*-OTU01 comprises *bamA* sequences with 92-93% nucleotide identity to *bamA* of *Sulfuritalea hydrogenivorans* sk43H (7 of 23 clones). The *bamA*-OTU02 includes sequences, which are distantly related to *S. hydrogenivorans* sk43H with 82-84% nucleotide identity (9 of 23 clones). The *bamA*-OTU03 comprises sequences with 89% nucleotide sequence identity to *bamA* of *Ramlibacter tataouinensis* strain 5-10 (7 of 23 clones). No sequences were affiliated to members of the genus *Georgfuchsia*, which dominated the initial *p*-xylene-degrading enrichment culture. Sequences of *bamA*-OTU01 and *bamA*-OTU02 were already detected in the initial culture, but with lower abundance (Sperfeld *et al.*, 2018). When cultivating pMB18 with benzoate in place of *p*-methylbenzoate, the *bamA* community changed. With benzoate, the majority of sequences belonged to the *bamA*-OTU04 (18 of 19 clones), which had 95% nucleotide identity to *bamA* of "*Aromatoleum aromaticum*" strain EbN1. Only a single sequence matching the *bamA*-OTU01 was detected.

Identification of a 4-MBCR gene cluster

As described above, for the dearomatization of *p*-alkylated substrates a specific benzoyl CoA reductase (4-MBCR) is required. A gene cluster putatively encoding a 4-MBCR was previously identified in the *p*-xylene-degrading enrichment culture obtained from the gasworks site (GenBank accession number: LT934314; Sperfeld *et al.*, 2018) and also found with 100% nucleotide sequence identity in culture pMB18 grown with *p*-methylbenzoate. This finding indicates that the microorganism harboring the 4-MBCR gene cluster was retained during the isolation procedure. When culture pMB18 was cultivated with benzoate, the PCR amplification of the 4-MBCR gene fragment failed (data not shown).

Discussion

A *p*-xylene-degrading microbial community dominated by *Georgfuchsia* sp. was previously enriched from a gasworks site in Thuringia (Sperfeld *et al.*, 2018). Isolation attempts using *p*-xylene as growth substrate failed so far. Therefore, we decided to replace *p*-xylene by the

water-soluble and less toxic *p*-methylbenzoate. Surprisingly, this procedure resulted in the loss of *Georgfuchsia* sp. Microorganisms related to *Sulfuritalea*, *Comamonadaceae* and *Ignavibacterium* became abundant. The detection of the *bamA* gene, which is a functional marker of the benzoyl-CoA pathway points to *Sulfuritalea* and *Comamonadaceae* as aromatic-compound degrading bacteria (ACDB) in this community. Organisms related to *Comamonadaceae* are frequently associated with the utilization of aromatic compounds (Blümel *et al*., 2001; Vacca *et al.*, 2005; Fahy *et al.*, 2006; Risso *et al.*, 2009; Yagi *et al*., 2009; Kim *et al*., 2010; Satola *et al*., 2013). *Sulfurtitalea* sp. are well known as sulfur-oxidizing bacteria that can grow under chemolithoautotrophic conditions (Kojima and Fukui, 2011; Watanabe *et al.*, 2014; Herrmann *et al.*, 2017). They were frequently detected in fresh-water habitats (Biderre-Petit *et al.*, 2011; Ferrer *et al.*, 2011; Kojima *et al.*, 2014; Lau *et al*., 2016; Zeng *et al.*, 2016), but also at hydrocarbon-polluted sites (Yagi *et al.*, 2010; Martin *et al.*, 2012; Guo *et al.*, 2017; Sperfeld *et al.*, 2018). To date, a possible contribution of *Sulfuritalea*-related microorganisms in aromatic compound degradation is rarely discussed. However, recent studies revealed that *S. hydrogenivorans* sk43H, which is the only isolated representative within this genus (Kojima and Fukui, 2011), anaerobically degrades carboxylic aromatic compounds with nitrate (Sperfeld *et al.*, unpublished). Furthermore, a microorganism closely related to strain sk43H, originally assigned to *Denitratisoma*, was enriched with *p*-xylene under nitrate reducing-conditions (Rotaru *et al*., 2010; Kojima and Fukui, 2011). The presence of *Sulfuritalea* in the *p*-methylbenzoate-degrading culture pMB18 further emphasizes its possible role as ACDB. The genus *Ignavibacterium* is not supposed to directly participate in aromatic compound degradation. Accordingly, no pathways for anaerobic aromatic compound degradation were found in the genome of *Ignavibacterium album* (Liu *et al.*, 2012). These fermentative bacteria were expected to thrive on dead biomass and/or metabolites of hydrocarbon degradation (Kleinsteuber *et al.*, 2012; Taubert *et al.*, 2012).

Culture pMB18 showed a specific shift in the composition of the microbial community when *p*-alkylated substrates were replaced by non-*p*-alkylated compounds. Members of the genus *Azoarcus* became abundant. In addition, considerable amounts of nitrite were formed. *Azoarcus* sp. are well-known as nitrate-reducing aromatic-compound degraders. They were frequently enriched with various aromatic compounds from different hydrocarbon-contaminated sites (summarized in Widdel *et al.*, 2000; Kleinsteuber *et al*., 2012; Lueders, 2016). Members of this genus were also described to excrete high amounts of nitrite into the environment (Braun and Gibson, 1984; Rabus and Widdel, 1995). The prevalence of *Azoarcus* sp. in culture pMB18 (when cultivated with non-*p*-alkylated compounds) was probably favored by the excretion of nitrite: up to 5 mM of nitrite were

formed in culture pMB18 dominated by *Azoarcus* sp. (Tab. 1, Fig. S2), but concentrations less than 1 mM already inhibited the *Sulfuritalea*/*Comamonadaceae* community presumably involved in degradation of *p*-alkylated substrates (Fig. S3). It seems reasonable that ACDB, which are inhibited by *Azoarcus*-dependent nitrite excretion, specialize on substrates that cannot be degraded by this microorganism (i.e. *p*-alkylated carboxylates). A specialization on *p*-alkylated monoaromatics requires a specific *p*-methylbenzoyl-CoA reductase (4-MBCR), the key-enzyme for the anaerobic dearomatization of *p*-alkylated aromatic ring systems (Lahme *et al.*, 2012). A putative 4-MBCR-coding gene region was found in the initial *p*-xylene degrading enrichment culture dominated by *Georgfuchsia* sp. (Sperfeld *et al.*, 2018) and was also detected in the *p*-methylbenzoate degrading culture pMB18. This study demonstrated, how the composition of a microbial community is guided by the aromatic substrate applied.

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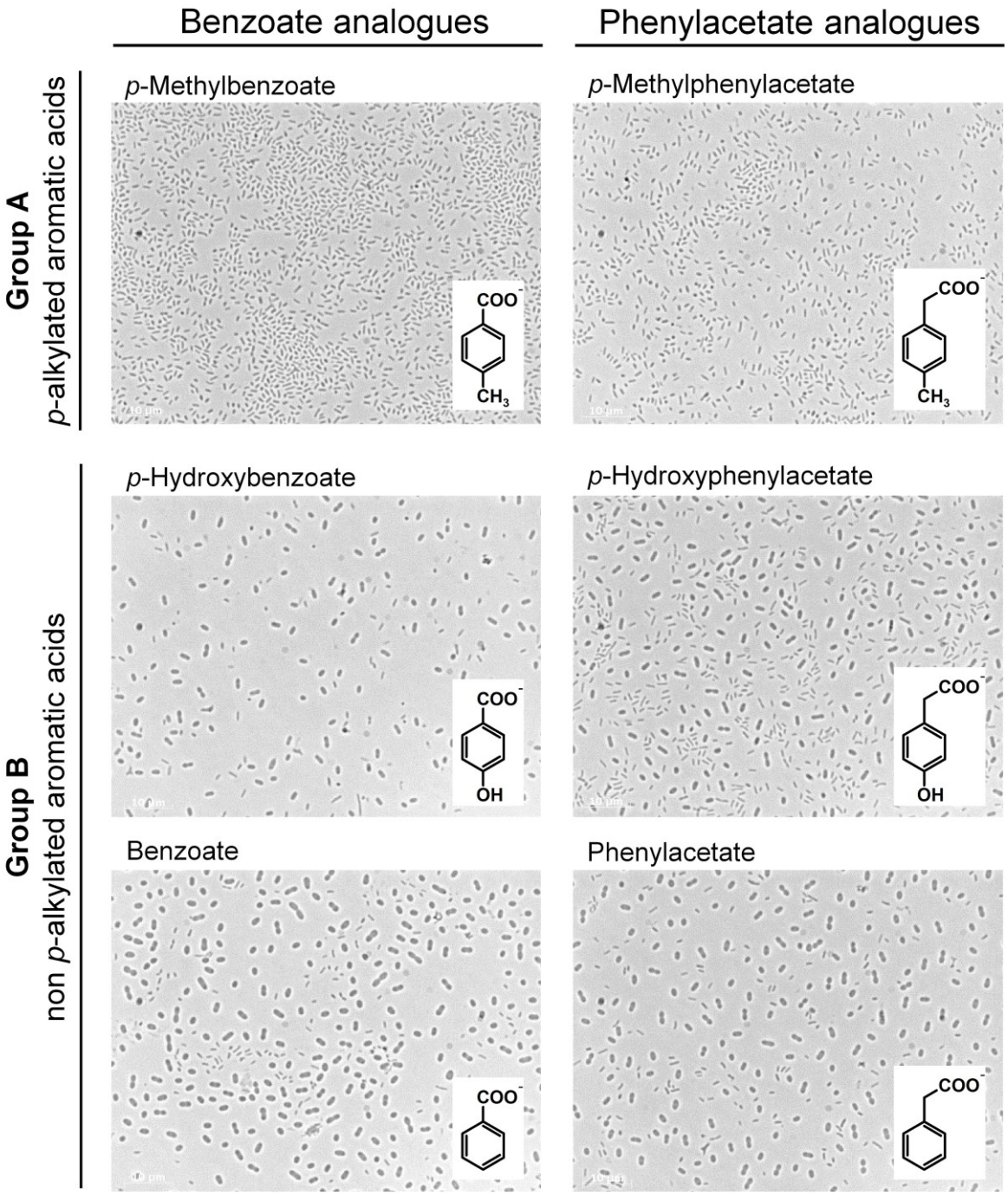

Figure 1: Microphotographs of culture pMB18 grown with selected *p*-alkylated (Group A) and non-*p*-alkylated aromatic compounds (Group B). Magnification x1,000.

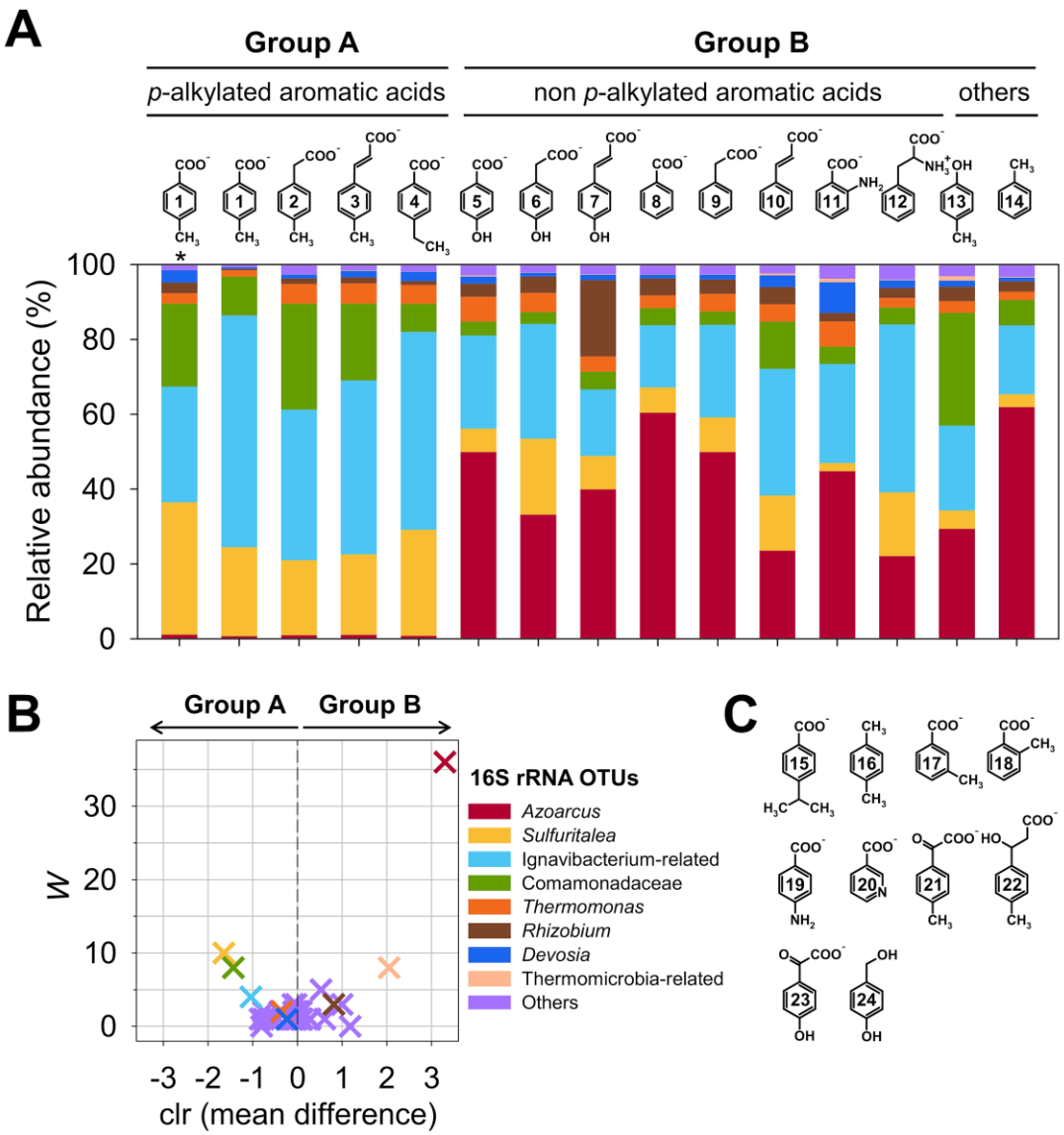

Figure 2: **(A)** Substrate-dependent changes in the bacterial community composition of culture pMB18 analyzed by deep 16S rRNA sequencing. Group A comprised aromatic compounds with an alkyl group in *para* position with respect to the carboxyl moiety. Group B comprised non-*p*-alkylated aromatic carboxylates including toluene and *p*-cresol. Culture pMB18 grown with *p*-methylbenzoate and nitrate was used as inoculum (marked with an asterisk). **(B)** Differential abundance analysis conducted with ANCOM. The analysis counts how often the abundance of a single OTU significantly differs from the abundance of the remaining OTUs upon cultivation with either group A or group B compounds (*W*). The *W*-values were plotted against logarithmized and centered differences in mean relative abundances of each OTU (*clr*). **(C)** Chemical structures of compounds not degraded by culture pMB18 (15-20) and of intermediates formed upon cultivation with *p*-methylphenylacetate, *p*-methylcinnamate, *p*-hydroxyphenylacetate, and cinnamate (21-24).

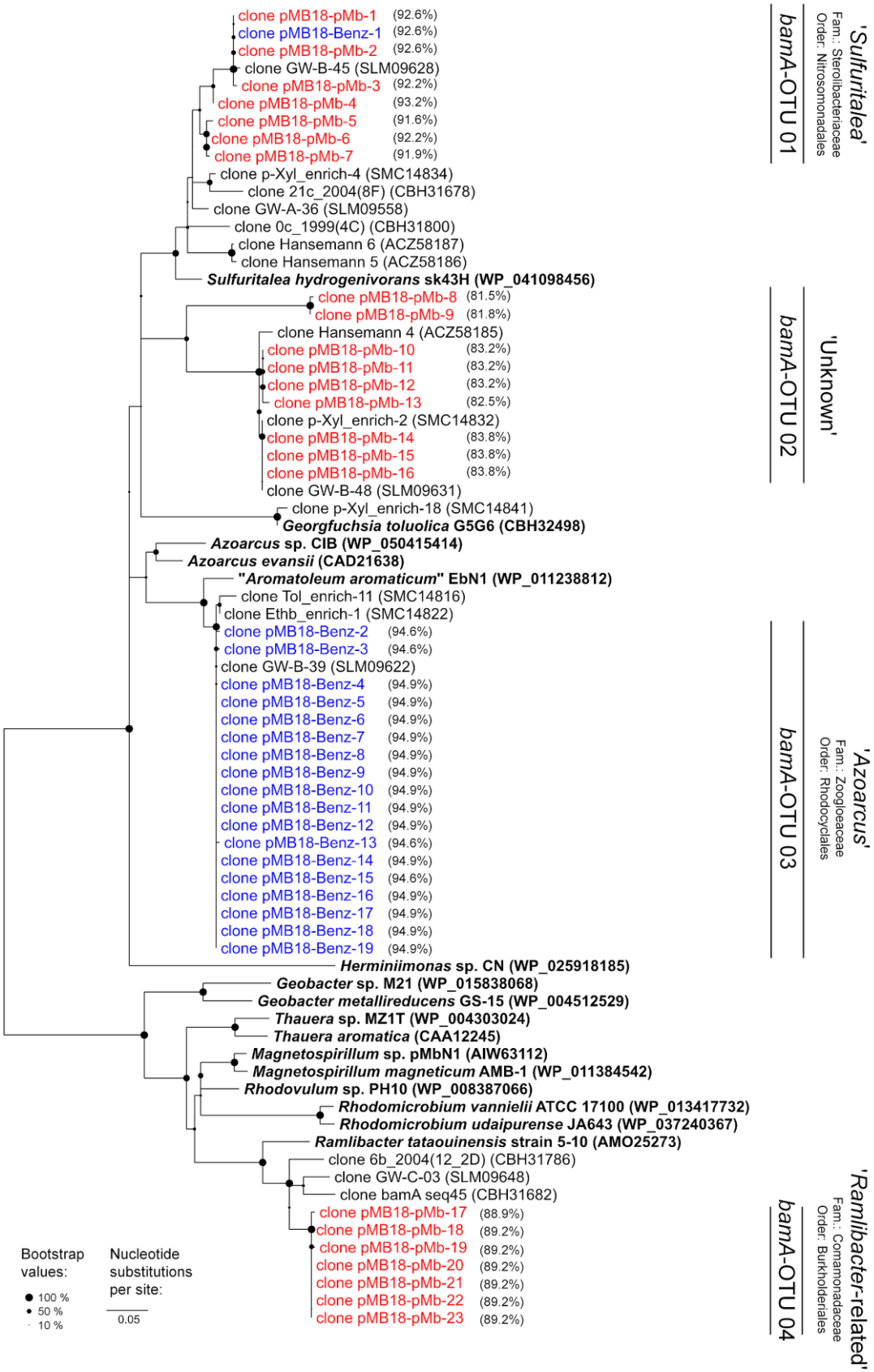
clone pMB18-pMb-1 (92.6%)
clone pMB18-Benz-1 (92.6%)
clone pMB18-pMb-2 (92.6%)
clone GW-B-45 (SLM09628)
clone pMB18-pMb-3 (92.2%)
clone pMB18-pMb-4 (93.2%)
clone pMB18-pMb-5 (91.6%)
clone pMB18-pMb-6 (92.2%)
clone pMB18-pMb-7 (91.9%)
clone p-Xyl_enrich-4 (SMC14834)
clone 21c_2004(8F) (CBH31678)
clone GW-A-36 (SLM09558)
clone 0c_1999(4C) (CBH31800)
clone Hansemann 6 (ACZ58187)
clone Hansemann 5 (ACZ58186)
Sulfuritalea hydrogenivorans sk43H (WP_041098456)
clone pMB18-pMb-8 (81.5%)
clone pMB18-pMb-9 (81.8%)
clone Hansemann 4 (ACZ58185)
clone pMB18-pMb-10 (83.2%)
clone pMB18-pMb-11 (83.2%)
clone pMB18-pMb-12 (83.2%)
clone pMB18-pMb-13 (82.5%)
clone p-Xyl_enrich-2 (SMC14832)
clone pMB18-pMb-14 (83.8%)
clone pMB18-pMb-15 (83.8%)
clone pMB18-pMb-16 (83.8%)
clone GW-B-48 (SLM09631)
clone p-Xyl_enrich-18 (SMC14841)
Georgfuchsia toluolica G5G6 (CBH32498)
Azoarcus sp. CIB (WP_050415414)
Azoarcus evansii (CAD21638)
"Aromatoleum aromaticum" EbN1 (WP_011238812)
clone Tol_enrich-11 (SMC14816)
clone Ethb_enrich-1 (SMC14822)
clone pMB18-Benz-2 (94.6%)
clone pMB18-Benz-3 (94.6%)
clone GW-B-39 (SLM09622)
clone pMB18-Benz-4 (94.9%)
clone pMB18-Benz-5 (94.9%)
clone pMB18-Benz-6 (94.9%)
clone pMB18-Benz-7 (94.9%)
clone pMB18-Benz-8 (94.9%)
clone pMB18-Benz-9 (94.9%)
clone pMB18-Benz-10 (94.9%)
clone pMB18-Benz-11 (94.9%)
clone pMB18-Benz-12 (94.9%)
clone pMB18-Benz-13 (94.6%)
clone pMB18-Benz-14 (94.9%)
clone pMB18-Benz-15 (94.6%)
clone pMB18-Benz-16 (94.9%)
clone pMB18-Benz-17 (94.9%)
clone pMB18-Benz-18 (94.9%)
clone pMB18-Benz-19 (94.9%)
Herminiimonas sp. CN (WP_025918185)
Geobacter sp. M21 (WP_015838068)
Geobacter metallireducens GS-15 (WP_004512529)
Thauera sp. MZ1T (WP_004303024)
Thauera aromatica (CAA12245)
Magnetospirillum sp. pMbN1 (AIW63112)
Magnetospirillum magneticum AMB-1 (WP_011384542)
Rhodovulum sp. PH10 (WP_008387066)
Rhodomicrobium vannielii ATCC 17100 (WP_013417732)
Rhodomicrobium udaipurense JA643 (WP_037240367)
Ramlibacter tataouinensis strain 5-10 (AMO25273)
clone 6b_2004(12_2D) (CBH31786)
clone GW-C-03 (SLM09648)
clone bamA seq45 (CBH31682)
clone pMB18-pMb-17 (88.9%)
clone pMB18-pMb-18 (89.2%)
clone pMB18-pMb-19 (89.2%)
clone pMB18-pMb-20 (89.2%)
clone pMB18-pMb-21 (89.2%)
clone pMB18-pMb-22 (89.2%)
clone pMB18-pMb-23 (89.2%)
Bootstrap values:
100 %
50 %
10 %
Nucleotide substitutions per site:
0.05
'Sulfuritalea'
Fam.: Sterolibacteriaceae
Order: Nitrosomonadales
bamA-OTU 01
'Unknown'
bamA-OTU 02
'Azoarcus'
Fam.: Zoogloeaceae
Order: Rhodocyclales
bamA-OTU 03
'Ramlibacter-related'
Fam.: Comamonadaceae
Order: Burkholderiales
bamA-OTU 04

Figure 3

Figure 3: Phylogenetic tree of *bamA* sequences detected in culture pMB18 upon cultivation with either *p*-methylbenzoate (red) or benzoate (blue). The tree was constructed using the maximum likelihood methods and evaluated with 1,000 bootstrap replicates. Detected *bamA* sequences were grouped into OTUs based on nucleotide identities with reference sequences and clustering in the tree. The *bamA*-OTUs 01 and 02 had highest nucleotide sequence identity to *Sulfuritalea hydrogenivroans* sk43H (given as %-values in parenthesis). The *bamA*-OTU 03 had highest identity to "*Aromatoleum aromaticum*" EbN1. The *bamA*-OTU 04 was distantly related to *Ramlibacter tatouinensis*. Accession numbers of *bamA* reference sequences from pure cultures and environmental samples are given in parenthesis.

Table 1: Monoaromatic compounds utilized by culture pMB18.

Substrate	Structure [b]	Group	Substrate conversion rate (mM/d)	Nitrate reduction rate (mM/d)	Nitrite formation (mM/d)	Intermediates formed from aromatic substrate		
						Compound	(mM) [d]	Structure [b]
p-Methylbenzoate [a]	1	A	0.21	1.11	n.d.	-		
p-Methylbenzoate	1	A	0.20	0.83	n.d.	-		
p-Methylphenylacetate	2	A	0.24	0.86	n.d.	*p*-Methylphenylglyoxylate	0.2	21
p-Methylcinnamate	3	A	0.21	0.71	n.d.	3-Hydroxy-3-(4-methylphenyl)propanate [c] *p*-Methylbenzoate	~0.3 0.2	22 1
p-Ethylbenzoate	4	A	0.04	0.19	n.d.	-		
p-Hydroxybenzoate	5	B	0.29	2.07	1.48	-		
p-Hydroxyphenylacetate	6	B	0.29	2.03	0.98	*p*-Hydroxyphenylglyoxylate	0.1	23
p-Hydroxycinnamate	7	B	0.11	1.03	0.80	*p*-Hydroxybenzoate	0.6	5
Benzoate	8	B	0.33	3.60	2.47	-		
Phenylacetate	9	B	0.28	2.04	0.70	-		
Cinnamate	10	B	0.13	1.41	0.87	Benzoate	0.2	8
Anthranilate	11	B	0.03	0.20	0.15	-		
Phenylalanine	12	B	0.08	0.51	0.16	-		
p-Cresol	13	B	0.16	1.74	1.62	*p*-Hydroxybenzoate *p*-Hydroxybenzyl alcohol	0.3 0.04	5 24
Toluene	14	B	0.02	0.13	0.12	-		

[a] Starting culture [b] Chemical structures are given in Figure 2 [c] Determined by mass spectrometry, estimated concentration [d] Maximum concentration detected.

n.d. = not detected (below detection limit)

Table 2: Abundant 16S rRNA sequences detected in culture pMB18 and affiliation to operational taxonomic units (OTU).

Sequences within 16S rRNA-OTUs	NCBI blast hit (cultivated organisms)	GenBank Acc. Number and position of NCBI blast hit		Query length	Coverage	Nucleotide identity (%)	Feature ID [a]
Azoarcus							
Sequence 1	*Azoarcus* sp. HxN1	AF331975.1	265..691	427	427	100	02_a
Sulfuritalea							
Sequence 1	*Sulfuritalea hydrogenivorans* sk43H	AP012547.1	1,067,637..1,068,063	427	417	97.7	03_a
Sequence 2	*Sulfuritalea hydrogenivorans* sk43H	AP012547.1	1,067,637..1,068,063	427	415	97.2	03_b
Ignavibacterium-related							
Sequence 1	*Ignavibacterium album* JCM 16511	CP003418.1	795,111..795,532	422	401	95.0	01_a
Comamonadaceae							
Sequence 1	*Xenophilus* sp. M2T2B11	GQ246689.1	332..758	427	424	99.3	04_a
Sequence 2	*Acidovorax* sp. sk40	KM056759.1	343..769	427	427	100	04_b
Sequence 3	*Simplicispira* sp. S33	KU233253.1	296..722	427	427	100	04_c
Thermomonas							
Sequence 1	*Thermomonas fusca* DSM 15424	AJ519986	331..757	427	427	100	05_a
Rhizobium							
Sequence 1	*Rhizobium selenitreducens* B1	NR_044216.1	320..721	402	402	100	06_a
Sequence 2	*Rhizobium* sp. VTT E-073064	EU438960.1	280..681	402	402	100	06_b
Devosia							
Sequence 1	*Devosia* sp. A16	CP012945.1	936,993..937,394	402	400	99.5	07_a
Thermomicrobia-related							
Sequence 1	*Sphaerobacter thermophilus* DSM 20745	CP001823.1	579,906..580,310	409	352	86.1	11_a

[a] Given in Table S1.

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3 OVERALL DISCUSSION

The here conducted experiments allowed a detailed insight into the microbial biodegradation processes that occur at the Thuringia gasworks site. Several research questions were proposed, which concerned the involvement of microorganisms and degradation pathways as well as the assessment of biodegradation processes. In the following, results are summarized and discussed, which were gained from the here applied culture-dependent and culture-independent approach. The focus lied on anaerobic ACDB that utilize BTEX compounds, since these organisms are expected to drive the major biodegradation processes at the Thuringia gasworks site.

3.1 POTENTIAL FOR ANAEROBIC AROMATIC COMPOUND DEGRADATION

As a first step, the microbial community present at the aquifer of the Thuringia gasworks site was investigated with a culture-independent approach by sequencing two marker genes; the universal 16S rRNA marker was aimed to detect the majority of bacteria, whereas the functional *bamA* marker was used to detect ACDB that possess strict anaerobic pathways for aromatic compound degradation. Based on the 16S rRNA analysis, several microorganisms were detected in the groundwater that are potentially able to degrade BTEX and other aromatic compounds under microaerobic, (low-ox ACDB) or strict anaerobic conditions (no-ox ACDB). The abundance of both ACDB groups was highest in the high contaminated groundwater well (44 % of 16S rRNA sequences) and decreased with decreasing BTEX concentrations. The majority of the low-ox ACDB taxa belonged to the betaproteobacterial family Comamonadaceae (i.e. *Rhodoferax*, *Polaromonas* and *Acidovorax*). These organisms are mainly described as oxygen-respiring bacteria, but also the utilization of alternative electron acceptors such as nitrate is feasible. For example, the Comamonadaceae organism *Rhodoferax ferrireducens* utilizes benzoate under Fe(III)- and nitrate-reducing conditions, but without possessing a strict anaerobic pathway for aromatic compound degradation (Risso *et al*., 2009). Similarly, aromatic compound-degrading members of the Comamonadaceae genera *Acidovorax* and *Polaromonas* were e.g. enriched under nitrate-reducing conditions with benzene or are adapted to low-oxygen concentrations, however, no anaerobic pathways were reported (Yagi *et al*., 2009; Fahy *et al*., 2016). The only Comamonadaceae bacterium that encodes an anaerobic degradation pathway for aromatic compounds is *Ramlibacter tatouinensis* (e.g. *bamA*, accession number: AMO25273), however, the degradation of aromatic compounds was not investigated in this organism (De Luca *et al*., 2009). The group of no-ox ACDB comprises bacteria that possess strict anaerobic degradation pathways and for which the oxygen-

independent utilization of aromatic compounds was observed. The majority of the no-ox ACDB detected in the gasworks aquifer belonged to the genera *Azoarcus*, *Sulfuritalea* (both Betaproteobacteria) and *Pelotomaculum* (Clostridia). *Azoarcus* spp. are model organisms for nitrate-dependent BTEX degradation and are frequently enriched or isolated from hydrocarbon polluted sites (Widdel *et al*., 2010). At the Thuringia gasworks site, members of *Azoarcus* were the highest abundant no-ox ACDB that were described before to degrade BTEX with nitrate. The role of *Sulfuritalea* spp. is ambiguous; they are recognized as sulfur-oxidizing chemolithoautotrophs, however, an involvement of these organisms in nitrate-dependent degradation of aromatic compounds is possible, as investigated in this work (see chapter 3.2). Members of the genus *Pelotomaculum* were reported to utilize BTEX compounds under fermenting conditions with syntrophic partners such as hydrogenotrophic sulfate-reducers (Kleinsteuber *et al*., 2012). Besides 16S rRNA analysis, the functional *bamA* gene was used as a second marker to specifically detect no-ox ACDB. The results from the 16S rRNA and *bamA* analyses were largely consistent; organisms related the no-ox ACDB *Azoarcus*, *Sulfuritalea* and *Pelotomaculum*, which were abundant according to 16S rRNA sequencing, were also frequently detected by *bamA* sequencing. In conclusion, anaerobic aromatic compound degradation seems to be well-established at the Thuringia gasworks site. Most of the low-ox and no-ox ACDB were affiliated to Betaproteobacteria, which are often facultative anaerobic bacteria that can either respire oxygen or alternative electron acceptors such as nitrate (Parales, 2010).

3.2 ANAEROBIC AROMATIC COMPOUND DEGRADATION IN *SULFURITALEA*

As deduced from 16S rRNA and *bamA* profiling, organisms related to *Sulfuritalea* were high abundant in the gasworks aquifer. The only isolated representative is *Sulfuritalea hydrogenivorans* sk43H, which is a chemolithoautotrophic organism that is recognized for its ability to oxidize sulfur and hydrogen under nitrate-reducing conditions (Kojima and Fukui, 2011; Kojima *et al*., 2014; Watanabe *et al*., 2014, 2016 & 2017). Organisms related to *Sulfuritalea* were frequently detected in various habitats, ranging from pristine carbon low groundwater to carbon rich activated sludge (Watanabe *et al*., 2017). In carbon low habitats, it was reported that *Sulfuritalea* spp. are important denitrifying sulfur oxidizers. For example, in a metatranscriptomics and metaproteomics study of an oligotrophic deep-subsurface aquifer, *Sulfuritalea* spp. were identified as chemolithoauthotrophic key players that drive the food web. Similarly, in studies that combined 16S rRNA profiling with *nirS* analysis (a functional marker for nitrite-reduction), it was found that *Sulfuritalea* spp. are high abundant, nitrate-reducing bacteria in pristine groundwater (Zeng *et al*., 2016; Herrmann *et al*., 2017). In the work presented here, it was hypothesized that *Sulfuritalea* spp. might also play a role

in the anaerobic degradation of aromatic compounds in carbon rich habitats. This was deduced from the reanalysis of several environmental studies where *Sulfuritalea* spp. were reported to be present. For example, a close relative of *Sulfuritalea hydrogenivorans* sk43H was enriched with *p*-xylene and nitrate (Rotaru *et al*., 2010). The enriched organism was originally assigned as a relative of *Denitratisoma oestradiolicum* (95 % 16S rRNA identity), however, reanalysis of the 16S rRNA gene revealed a closer relationship to *Sulfuritalea hydrogenivorans* sk43H (98% 16S rRNA identity; Kojima and Fukui, 2011). *Sulfuritalea*-related *bamA* sequences, which are indicative for anaerobic aromatic compound degradation, were detected in the aquifer of a landfill leachate, at a former benzene production plant and in diesel fuel polluted Antarctic soils (Staats *et al*., 2011; Sampaio *et al*. 2017; Kuntze *et al*., 2011). It was noted earlier that the denitrifying isolate *S. hydrogenivorans* sk43H utilizes the aromatic compound benzoate as electron donor, however, the PCR amplification of functional marker genes for anaerobic aromatic compound degradation failed and the genome was not investigated for involved pathways (Kojima *et al*., 2014). In line with this, an involvement of *Sulfuritalea* spp. in anaerobic degradation of aromatic compounds is rarely discussed. To emphasize the potential role of *Sulfuritalea* in anaerobic aromatic compound degradation, the genome of strain sk43H was reanalyzed and several aromatic compounds were tested for their utilization under nitrate-reducing conditions. It was found that *S. hydrogenivorans* sk43H encodes strict anaerobic pathways for the degradation of simple aromatic carboxylic acids and utilizes the aromatic compounds benzoate, 4-hydroxybenzoate, phenylacetate and 4-hydroxyphenalyacetate. These compounds are typically plant-derived, however they can be also released by no-ox ACDB during the degradation of industrially derived aromatic compounds such as BTEX, phenol or *p*-cresol (Carmona *et al*., 2009; Widhalm and Dudareva, 2015). The here conducted experiments provide a physiological and genetic basis for anaerobic aromatic compound degradation in *Sulfuritalea* and underlined the potential role of these organisms as no-ox ACDB at the Thuringia gasworks site.

3.3 *AZOARCUS* AND *GEORGFUCHSIA* UTILIZE BTEX WITH NITRATE

Experiments with microbial enrichment cultures were aimed to proof, which organisms detected at the Thuringia gasworks site are involved in nitrate-dependent BTEX degradation. In these enrichment cultures, BTEX utilization was observed for toluene, ethylbenzene, *p*-xylene and *m*-xylene, but not for *o*-xylene and benzene. Toluene- and ethylbenzene-degrading microorganisms were readily enriched from all three groundwater wells along the BTEX pollutant gradient, indicating that the enriched organisms have an active role in biodegradation. Sequencing of the 16S rRNA and *bamA* gene confirmed that

members of *Azoarcus* are involved in nitrate-dependent utilization of toluene and ethylbenzene. Microorganisms that utilize *p*-xylene were enriched from two of the three groundwater wells after a prolonged lag-phase. Gene marker analyses revealed that members of *Georgfuchsia* are involved in *p*-xylene degradation. *Georgfuchsia* spp. were less abundant in the gasworks aquifer than *Azarcus* spp, which might explain the observed prolonged lag-phases. *Georgfuchsia toluolica* G5G6 is the only isolated representative within this genus. This isolate degrades toluene and ethylbenzene as electron donors but it does not utilize *p*-xylene (Weelink *et al*., 2009). Strain G5G6 originated from an iron-reducing, BTEX polluted aquifer and it was the first BTEX-degrading Betaproteobacterium that utilized Fe(III) as electron acceptor. The utilization of Fe(III) was also shown for the here enriched *Georgfuchsia*-dominated culture (see Supplementary materials, chapter V, p. xxii, Fig. S1). In conclusion, it was confirmed that *Azoarcus* spp. are key players in nitrate-dependent BTEX degradation at the gasworks site. Further, nitrate-reducing *Georgfuchsia* spp. occurred in lower abundances, but were specialized for the degradation of the particularly recalcitrant compound *p*-xylene.

3.4 UTILIZATION OF *P*-ALKYLATED AROMATIC COMPOUNDS

The degradation of *p*-alkylated aromatic compounds such as *p*-xylene is of special interest for bioremediation, since these compounds are often reported to be particularly recalcitrant (Rabus *et al*., 2016). Unexpectedly, the utilization of *p*-xylene occurred with higher rates than the utilization of *m*- and *o*-xylene in enrichment cultures from the Thuringia gasworks site. This indicated a well-established potential for the degradation of *p*-alkylated aromatic compounds in the aquifer. The recalcitrance of these compounds arises from mechanistic constraints: The critical step for the degradation of *p*-alkylated compounds is the reductive dearomatization of *p*-alkylated benzoyl-CoA intermediates, which is sterically hindered in conventional class I benzoyl-CoA reductases (BCR) by the *p*-alkyl substituent. Therefore, the reductive dearomatization of e.g. 4-methylbenzoyl-CoA requires a specific 4-methylbenzoyl-CoA reductase (4-MBCR). Such an enzyme was found in the nitrate-reducing Alphaproteobacterium *Magnetospiillum* sp. pMbN1. To date, strain pMbN1 is the only pure culture that degrades *p*-methylbenzoate under anoxic conditions (Lahme *et al*., 2012). Likewise, no nitrate-reducing pure culture is available that utilizes *p*-xylene and only few isolates were reported that degrade *p*-alkylated aromatic compounds under anaerobic conditions (Strijkstra 2014; Higashioka *et al*., 2012). As an exception, the enrichment of a *p*-xylene degrading, denitrifying culture succeeded with samples from a wastewater treatment plant in Northern Germany (Rotaru *et al*., 2010). This enrichment culture was dominated by an organism related to *Sulfuritalea* (originally assigned as *Denitratisoma*-

related). Interestingly, *Sulfuritalea*-related organisms were among the most abundant taxa in the aquifer of the Thuringia gasworks site. They also co-occurred with *Georgfuchsia* in the nitrate-reducing, *p*-xylene-degrading enrichment culture (≈ 4% of 16S rRNA sequences) and were even further enriched when *p*-xylene was replaced by *p*-methylbenzoate (indicated by direct 16S rRNA amplicon sequencing, unpublished). Based on this, it was investigated, if *Sulfuritalea* spp. are potentially involved in the degradation of *p*-alkylated compounds. Therefore, a single anoxic isolation step was conducted on agar, using *p*-methylbenzoate as electron donor. This procedure failed to isolate *Sulfuritalea* sp., however, a new enrichment culture was obtained, which showed interesting phenotypic characteristics. In culture pMB18, *Georgfuchsia* sp. was absent and *Sulfuritalea*-related organisms (≈ 35% of 16S rRNA sequences) co-occurred with bacteria affiliated to Comamonadaceae (≈ 22%) and *Ignavibacterium* (≈ 31%). The *Sulfuritalea*/Comamonadaceae community was most likely involved in the degradation of *p*-methylbenzoate. In accordance, the majority of *bamA* sequences retrieved from this culture were affiliated to the same organisms. Bacteria related to *Ignavibacterium* were almost absent at the Thuringia gasworks site and are more likely involved in the degradation of cell debris or intermediary metabolites such as acetate (Kleinsteuber *et al*., 2012; Liu *et al*., 2012; Taubert *et al*., 2012). In a next step, it was analyzed which *p*-alkylated and non-*p*-alkylated aromatic compounds are utilized by culture pMB18. The analysis showed that culture pMB18 lost its ability to degrade *p*-xylene, which is reasonable since *Georgfuchsia* was absent after the “isolation” step. However, culture pMB18 utilized the *p*-alkylated aromatic compounds *p*-methylbenzoate, *p*-methylphenylacetate, *p*-methylcinnamate and *p*-ethylbenzoate. The degradation of these compounds was coupled to the reduction of nitrate to probably N_2 without intermediate formation of nitrite. The community composition of pMB18 was stable when *p*-alkylated compounds were used as substrate. A different phenotype was observed, when non-*p*-alkylated aromatic compounds such as benzoate, phenylacetate, cinnamate or toluene were used. With these substrates, the reduction of nitrate proceeded via an intermediate formation of almost equimolar amounts of nitrite (≈ 5 mM). Also a pronounced community shift was observed: The *Sulfuritalea*/Comamonadaceae community was suppressed and members of *Azoarcus* became abundant (increase from ≈ 1% to ≈ 62% of 16S rRNA sequences). The excretion of nitrite by *Azoarcus* seems to be a common characteristic for members this genus and was also reported for *Azoarcus* pure cultures (Braun and Gibson, 1984; Rabus and Widdel., 1995). It is likely that *Azoarcus* spp. excrete nitrite at the gasworks site, which has considerable ecological implications. *Azoarcus*-dependent nitrite-excretions inhibit the *Sulfuritalea*/Comamonadaceae ACDB community, as shown by the external addition of

nitrite to culture pMB18. At the Thuringia gasworks site, the *Sulfuritalea*/Comamonadaceae community might have evolved a strategy to avoid the competition with *Azoarcus* and the therewith accompanied inhibition by nitrite. By specializing on substrates that are not utilized by *Azoarcus*, the *Sulfuritalea*/Comamonadaceae community can settle in niches where nitrite concentrations are expected to be low. Such a specialization would require a specific 4-methylbenzoyl-CoA reductase (4-MBCR). In accordance, a putative 4-MBCR encoding gene cluster was PCR-amplified from a *Sulfuritalea*/Comamonadaceae-dominated pMB18 culture (grown with *p*-methylbenzoate), but not from a pMB18 culture dominated by *Azoarcus* (grown with benzoate). To further proof that *Azoarcus*-dependent nitrite-excretions drive the specialization of ACDB for substrates that are not utilized by *Azoarcus*, e.g. pure cultures of involved organisms are required.

3.5 PROFILING OF *BAMA* REVEALS ELECTRON-ACCEPTING PROCESSES

The detection of electron-accepting processes is a major concern for the application of bioremediation strategies (Christensen *et al*., 2000). In theory, the availability of electron acceptors governs the biodegradation activity, since more energy can be conserved form the reduction of thermodynamically more favorable electron acceptors. The most favorable electron acceptor is O_2, followed by nitrate, manganese, Fe(III), sulfate and CO_2. The utilization of a more favorable electron acceptor results in higher growth yields and growth rates. The recently proposed plume fringe concept generalizes the biodegradation processes that occur within contamination plumes (Meckenstock *et al*., 2015): If contaminants such as hydrocarbons are present in an excess, all available electron acceptors are consumed until an almost steady state situation is reached. In this situation, soluble electron acceptors such as oxygen, nitrate or sulfate are depleted in the plume center. In this center, biodegradation occurs via fermentation or by the reduction of insoluble Fe(III) or Mn(IV) minerals. Soluble electron acceptors are only replenished at the outer fringes of the plume from the surrounding “fresh” groundwater. As a result, most of the biodegradation process occur at these plume fringes and are slow in the plume center. However, it needs to be mentioned that process are dynamic and can shift due to fluctuating groundwater conditions (Anesser *et al*., 2008; Tischer *et al*., 2013; Meckenstock *et al*., 2015). In the work presented here, it was investigated, if electron-accepting processes can be predicted by analyzing the *bamA* gene marker. This was anticipated earlier: *bamA* sequences affiliated to Fe(III)-reducing *Geobacteraceae* were high abundant in an iron-rich aquifer, whereas *bamA* sequences affiliated to sulfate-reducing and/or fermenting Deltaproteobacteria and Firmicutes dominated anoxic oil wells (Staats *et al*., 2011; Verde *et al*., 2013; Ruan *et al*., 2016). At the Thuringia gasworks site, *bamA* analysis indicated

that different electron accepting processes occur along the pollutant gradient. In the high contaminated groundwater well, circa 44 % of the detected *bamA* sequences were affiliated to obligate anaerobic sulfate-reducing and/or fermenting members of the Deltaproteobacteria and Clostridia. Most of the other *bamA* sequences were affiliated to nitrate-reducing Betaproteobacteria (circa 37 %). Since sulfate was almost depleted in the high contaminated groundwater well, it can be concluded that sulfate- and/or CO_2-reduction are the predominating electron-accepting processes. The dominance of these processes requires the limitation of more favorable electron acceptors such as oxygen or nitrate (Meckenstock *et al.*, 2015). Further downstream of the high contaminated groundwater well, sulfate- and/or CO_2-reducing bacteria were almost absent and facultative anaerobic Betaproteobacteria dominated the microbial community (up to 92%). It seems that nitrate or oxygen were sufficiently available in this area of the plume, enabling facultative anaerobic Betaproteobacteria to outcompete sulfate-/CO_2-reducing bacteria. In all three groundwater wells, a minority of *bamA* sequences were affiliated to Fe(III)-reducing Geobacteraceae (≤ 15%), indicating that Fe(III)-dependent biodegradation is feasible, but less pronounced at the gasworks aquifer. In conclusion, analysis of the *bamA* diversity predicts that nitrate-reducing no-ox ACDB are well-established at the Thuringia gasworks site, however, their activity seems to be limited in the high contaminated groundwater well by an insufficient supply of nitrate. The analysis emphasizes the applicability of *bamA* as marker to predict electron-accepting processes.

3.6 BIOREMEDIATION WITH NITRATE AND ASSESSMENT OF BIODEGRADATION

Bioremediation strategies can be aimed to stimulate aerobic or anaerobic biodegradation processes. Aerobic bioremediation is promising at sites with a short history of e.g. BTEX exposure. These sites are not pre-conditioned to the biodegradation of pollutants and they profit from oxygen-dependent bacteria that quickly establish from small cell numbers (Herzyck *et al.*, 2014). Despite higher growth rates of aerobic ACDB, the bioremediation with oxygen has considerable drawbacks: Oxygen is only soluble in small quantities in water (≈ 8 mg/l) and it is often reported to cause the clogging of injection wells by rapid biomass formation or precipitation of e.g. iron oxides (Farhadian *et al.*, 2008). As an alternative to oxygen-dependent bioremediation, anaerobic bioremediation with e.g. nitrate is feasible (Morgan *et al.*, 1993; Broholm and Arvin, 2000; Spence *et al.*, 2001; Rivett *et al.*, 2008). Anaerobic biodegradation occurs with slower rates and anaerobic communities take longer to establish. Therefore, an anaerobic bioremediation strategy is more suitable at sites that are pre-conditioned for anaerobic biodegradation after a long-term exposure to pollutants. Anaerobic bioremediation with nitrate is a promising alternative to oxygen-dependent

biodegradation. Nitrate has a higher solubility in water (≈ 800 g/l, the injection is restricted to 50 mg/ml) and it belongs to the thermodynamically more favorable electron acceptors which is widely utilized by diverse bacteria (Simon and Klotz, 2013). As another advantage, the addition of nitrate causes the abiotic re-oxidation of toxic sulfide to sulfate in sulfate-reducing areas of contamination plumes, making sulfate available again for sulfate-dependent biodegradation (Eckert and Apello, 2002). Several reports about the implementation of nitrate-dependent bioremediation strategies are available (Curtis and Lammey, 1998; Dybas *et al*., 1998; Hutchins *et al*., 1991 and 1998; Cunningham *et al*., 2001; Eckert and Apello, 2002; Xu *et al*., 2015). As a major obstacle for nitrate-dependent bioremediation, it is uncertain if polluted sites are sufficiently pre-conditioned for nitrate-dependent biodegradation. In this work, two marker genes were combined to assess the potential for nitrate-dependent aromatic compound degradation at the Thuringia gasworks site. Based on this culture-independent approach, it was concluded that nitrate-reducing ACDB are well-established along the pollutant gradient, however, their activity seemed to be limited in high contaminated, sulfate-/CO_2-reducing groundwater by an insufficient supply of nitrate (see chapter 3.5, p. 68). Therefore, nitrate-dependent stimulation of biodegradation processes is suggested for the sulfate-/CO_2-reducing area of the contamination plume.

At the Thuringia gasworks site, the applicability of nitrate-dependent bioremediation was shown by using nitrate-reducing microbial enrichment cultures. In these cultures, biodegradation was feasible for the BTEX compounds toluene, ethylbenzene, *p*-xylene and *m*-xylene, but not for *o*-xylene and the particularly recalcitrant compound benzene. In principle, benzene degradation is feasible with nitrate, as shown by e.g. the enrichment of a nitrate-reducing benzene-degrading culture from a gasoline station (Johnson *et al*., 2003; Luo *et al*., 2014). This culture was dominated by organisms related to *Azoarcus* and Peptococcaceae, which seemed to degrade benzene in a syntrophic interaction. According to 16S rRNA and *bamA* sequencing, organisms related to *Azoarcus* and Peptococcaceae (i.e. *Pelotomaculum*) were also abundant at the Thuringia gasworks site. The inability to enrich benzene-degrading cultures from the Thuringia gasworks site does not necessarily mean that nitrate-dependent benzene degraders are absent. It is possible that the conditions chosen for the microbial enrichments were unfavorable. For example, the enrichment cultures were incubated under continuous shaking, however, reports indicate that shaking may disturb syntrophic interactions possibly involved in nitrate-dependent benzene degradation (Dannenberg *et al*., 1997; Vogt *et al*., 2011). To proof *in situ* benzene biodegradation, the use of e.g. BACTRAP® microcosms might be applicable (explained in chapter 1.4, page 7).

As outlined above, the assessment of microbial biodegradation is an important prerequisite to plan or monitor bioremediation strategies. Elaborate methods are available that combine e.g. multi “omics” approaches or stable isotope probing (SIP) to study underlying food webs with high resolution (e.g. Taubert *et al*., 2012; An *et al*., 2013; Callaghan *et al*., 2013; Tan *et al*., 2015; Anantharaman *et al*., 2016; Lau *et al*., 2016). However, these methods are often time-consuming and are not yet applicable as routine diagnostic tool. In recent year, PCR-based analyses of universal and functional marker genes gained increasing popularity to asses biodegradation processes and hundreds of possible primer pairs were described (reviewed by e.g. Fischer *et al*., 2016; Imhoff, 2016; von Netzer *et al*., 2016). To assess anaerobic aromatic compound degradation, the genes encoding for three different enzymatic reactions are currently targeted: (i) *bssA*, (ii) *bcr*/*bzd* and *bamB* and (iii) *bamA* (see Introduction, p. 4, Fig. 1). The aim of the here presented work was to detect phylogenetically diverse bacteria and key-processes involved in biodegradation at gasworks site by keeping the approach applicable for routine diagnostic. These demands were covered by the combined analysis of the 16S rRNA and *bamA* marker genes. The analysis of the 16S rRNA gene revealed a broad overview of the majority of bacteria and allowed to identify organisms that were previously reported to degrade aromatic compounds under different electron-accepting processes. In a similar study, a 16S rRNA-based approach identified sulfate- and CO_2-reducing bacteria that dominated different areas of a hydrocarbon plume (Tischer *et al*., 2013). Since the 16S rRNA-detected bacteria do not necessarily possess the genes for anaerobic aromatic compound degradation, the *bamA* gene was chosen as an additional functional marker to specifically detect no-ox ACDB. As a major advantage, the *bamA* gene is conserved in facultative anaerobic and obligate anaerobic bacteria and a single primer set is sufficient to detect members from phylogenetically diverse groups (Kuntze *et al*., 2008; Staats *et al*., 2011). A second *bamA* primer set was reported to detect additional sulfate-reducing no-ox ACDB (Kuntze *et al*. 2011), however, this primer set failed to amplify the *bamA* gene from the Thuringia gasworks site (unpublished). The *bamA* analysis is limited by the availability of *bamA* reference sequences of characterized bacterial pure cultures or enrichment cultures. Some of the environmental *bamA* sequences retrieved from the gasworks site were roughly affiliated to a respiratory guild (e.g. nitrate-reduction vs. sulfate/CO_2-reduction), but not to a specific genus. For example, uncultured *bamA* sequences retrieved from the sulfate-/CO_2-reducing high contaminated area of the plume were affiliated to “obligate anaerobic sulfate-reducing and/or fermenting members of the Deltaproteobacteria and Clostridia”, but not to a bacterial genus or family. These uncultured *bamA* sequences had only 77 – 82% nucleotide sequence identity to *bamA* sequences of cultivated reference organisms. It cannot be

concluded, if e.g. the here detected clostridial *bamA* sequences belonged to the genera *Pelotomaculum*, *Desulfotomaculum* (both Peptococcaceae family) or even to a different family. However, for the assessment of biodegradation processes, an affiliation of *bamA* sequences to respiratory guilds is sufficient to assess relative differences in the potential for anaerobic aromatic compound degradation between two or more sampling wells. Results from 16S rRNA and *bamA* analyses were largely consistent between environmental samples and enrichment cultures. For example, both marker genes were suitable to detect no-ox ACDB belonging to facultative anaerobic Betaproteobacteria including *Azoarcus*, *Sulfuritalea*, *Georgfuchsia* and Comamonadaceae, as well as to obligate anaerobic Clostridia and Deltaproteobacteria.

For the analysis of 16S rRNA and *bamA* gene diversity, two different methods were chosen: next-generation amplicon sequencing and clone library sequencing. The prices for next-generation sequencing dropped drastically after the introduction of paired-end sequencing on Illumina MiSeq platforms (Tan *et al*., 2015). In contrast, clone-library sequencing is more laborious and the resolution is poor; i.e. the number of sequence reads analyzed by deep 16S rRNA sequencing and *bamA* clone library sequencing differed by a factor of circa 1,000. For a future application of *bamA* analysis as routine-diagnostic tool, an approach based on next-generation sequencing is highly recommended.

In conclusion, the here conducted work gave an unprecedented insight into the biodegradation processes that occur in the aquifer of a former gasworks sites. This was the first thorough characterization of a gasworks aquifer by combining the 16S rRNA and *bamA* marker in environmental samples and enrichment cultures. A future application of these two marker genes for a routine diagnostic approach to assess biodegradation processes at aromatic compound polluted sites is suggested. The conducted experiments contribute to a better understanding of anaerobic aromatic compound-degrading communities and their underlying ecological principles.

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V SUPPLEMENTARY MATERIALS

Supplement Manuscript I

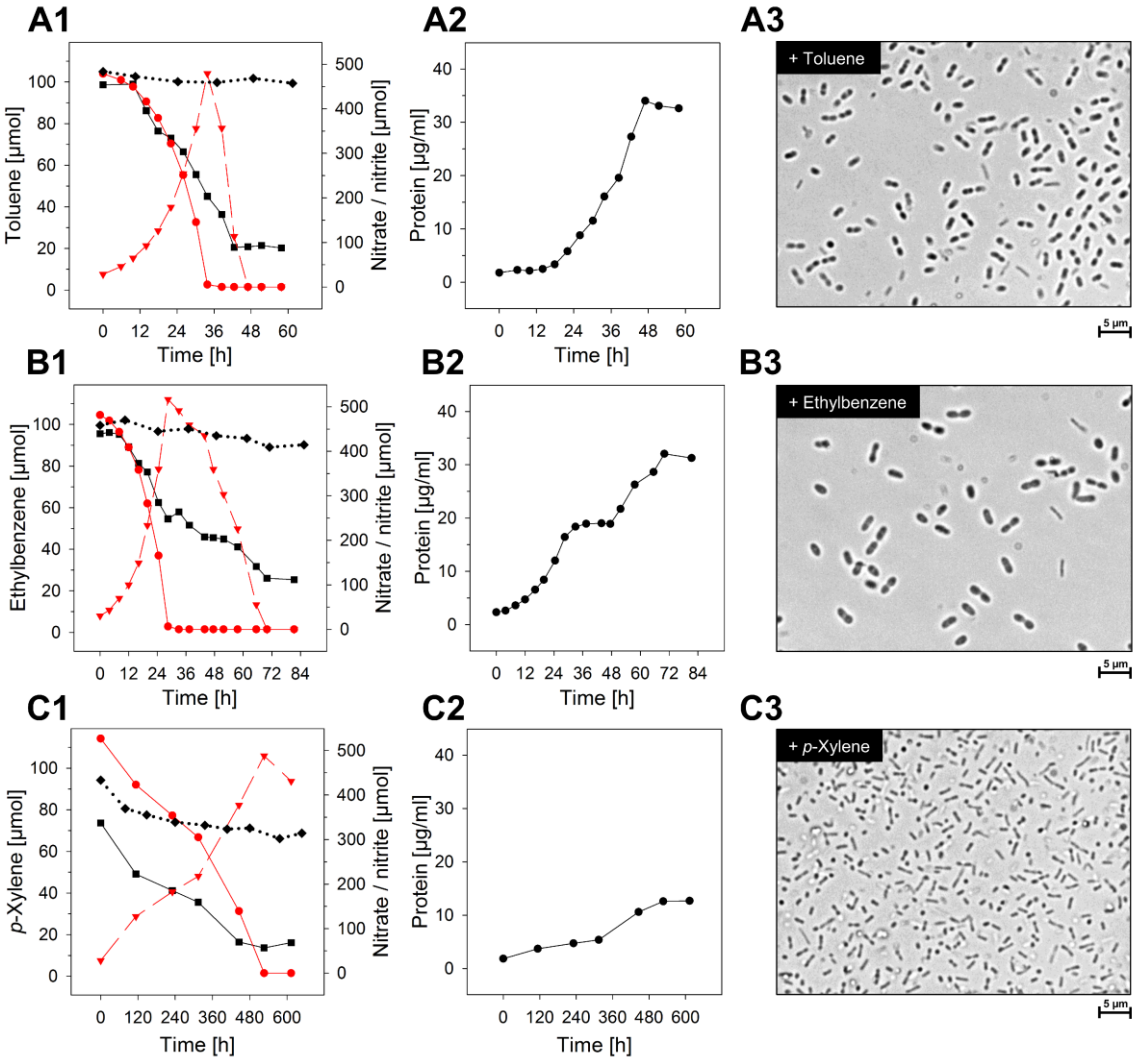
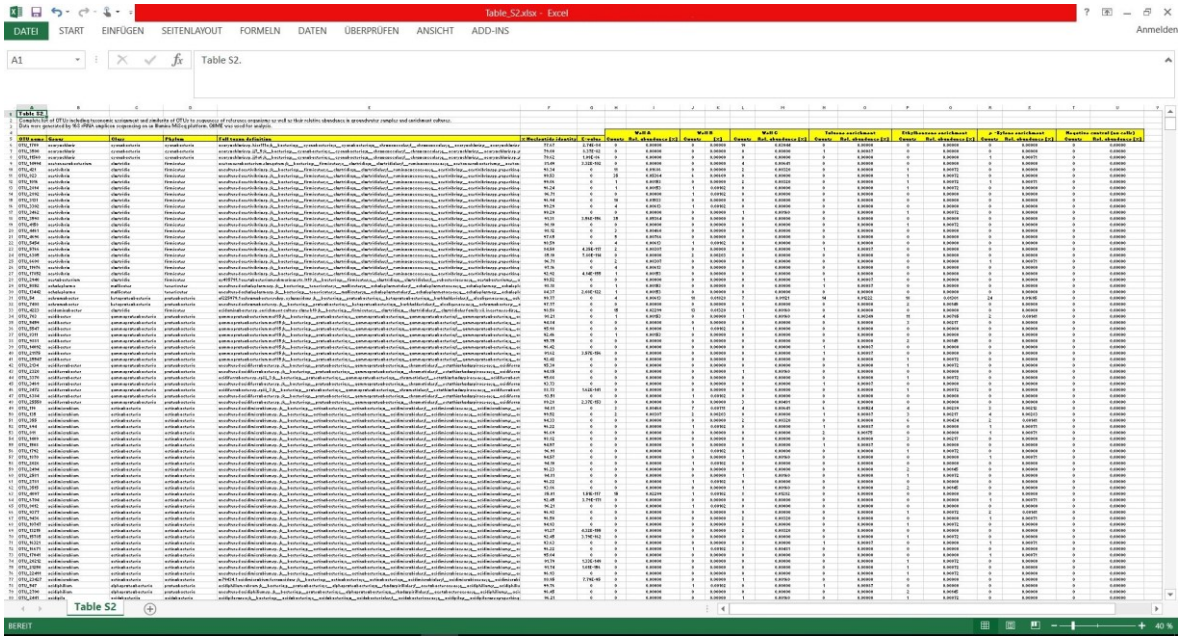


Figure S1: Substrate conversion, growth and microphotographs of microbial cultures enriched with toluene (**A**), ethylbenzene (**B**) or *p*-xylene (**C**). A1-C1: Consumption of BTEX (black filled squares) with nitrate (red filled circles) and formation of nitrite (red inverted triangle). The dotted line with filled diamonds indicates the loss of BTEX in control experiments without cells. A2-C2: The protein concentration (black filled circles) was plotted as indicator of bacterial growth. A3-C3: Microphotographs of BTEX-degrading enrichment cultures (1000-fold magnification, bright field).

Table S1: Groundwater parameters determined for sampling wells A, B, and C.

Groundwater parameter/ Sampling well	Date of sampling														
	08/30/2012	*02/15/2013*	*03/14/2013*	*05/07/2013*	*06/19/2013*	*07/18/2013*	*08/16/2013*	*09/13/2013*	*10/29/2013*	*11/22/2013*	*12/12/2013*	*01/16/2014*	*Median*	*Minimum*	*Maximum*
***Oxygen** [mg/l]*															
Well A	0.6												0.6		
Well B		0.2		0.3	0.1	0.1	0.1	0.1	0.1	1.5	1.8		0.1	0.1	1.8
Well C	0.1	0.1	0.2	0.1	0.3	1.4	1.0	0.2	0.2	1.5	0.1		0.2	0.1	1.5
***Nitrate** [mg/l]*															
Well A	13.1												13.1		
Well B											0.0	2.7	1.4	0.0	2.7
Well C	1.4		0.0	2.9			2.3	1.0	1.8	0.0	0.6	1.0	1.0	0.0	2.9
***Ammonium** [mg/l]*															
Well A	23.7												23.7		
Well B											14.8	14.9	14.9	14.8	14.9
Well C	5.1						4.9	5.1	3.6	4.7	5.2	5.7	5.1	3.6	5.7
***Sulfate** [mg/l]*															
Well A	71												71		
Well B											300	334	317	300	334
Well C	475		561	639			647	605	571	559	541	497	561	475	647
***Iron** (soluble) [mg/l]*															
Well A	< 0.1												< 0.1		
Well B											1.2	0.2	0.7	0.2	1.2
Well C	1.0						0.3		5.6	1.6	2.1	11.5	1.9	0.3	11.5
***BTEX** [µg/l]*															
Well A	9842												9842		
Well B		1943	1321	716	825	11	357	1651	2725	3296	3136	2031	1651	11	3296
Well C	143	263	174	29	16	46	122	208	308	390	309	128	158	16	390
***Hydrocarbon oil index** [µg/l]*															
Well A	4300												4300		
Well B		0	350	580	440	290	180	300	770	450	210	280	300	0	770
Well C	600	450	270	0	0	130	150	270	150	220	100	100	150	0	600
***Phenol index** [µg/l]*															
Well A	153												153		
Well B		0	81	65	90	126	186	91	134	188	275	82	91	0	275
Well C	31	41	26	0	15	17	37	38	29	28	38	26	29	0	41
***PAH** [µg/l]*															
Well A	63												63		
Well B		67	128	93	132	111	67	101	104	135	127	108	108	67	135
Well C	25	50	22	11	13	18	15	4	28	34	33	33	23	4	50
***Hydrogen carbonate** [mg/l]*															
Well A	805												805		
Well B											645	641	643	641	645
Well C	533		544	536					517	510	518	514	518	510	544
***Electrical conductivity** [µS/cm]*															
Well A	1629												1629		
Well B		2030	2160	1349	2100	2060	2080	2010	502	1855	1787		2020	502	2160
Well C	1743	1768	1722	1242	2020	2120	2060	1952	437	1945	1880		1880	437	2120
pH-value															
Well A	6.3												6.3		
Well B		8.0	6.8	6.9	7.1	7.2	7.3	6.8	7.0	6.9	7.0		7.0	6.8	8.0
Well C	6.8	8.6	9.2	7.0	7.1	7.2	7.2	6.8	6.9	6.9	6.8		7.0	6.8	9.2
***Redox potential** [mV]*															
Well A	-130												-130		
Well B		-62	-5	+13	-105	-108	-121	-128	-197	-130	-69		-107	-197	+13
Well C	-124	-78	-151	+77	-122	-90	-97	-107	-145	-78	-105		-105	-151	+77
***Temperature** [°C]*															
Well A	14.6												14.6		
Well B		10.7	4.9	12.5	13.4	12.7	12.2	12.2	12.3	11.5	8.2		12.2	4.9	13.4
Well C	15.1	10.7	10.1	10.0	11.2	11.0	11.7	13.7	12.3	10.2	10.4		11.0	10.0	15.1

Table S2: Complete list of OTUs including taxonomic assignment and similarity of OTUs to sequences of reference organisms as well as their relative abundance in groundwater samples and enrichment cultures. Data were generated by 16S rRNA amplicon sequencing on an Illumina MiSeq platform. QIIME was used for analysis.



For full table, see supplemental CD-ROM (./Manuscript_I/Table_S2.xlsx) or download from: https://ars.els-cdn.com/content/image/1-s2.0-S0043135417310357-mmc2.xlsx

Table S3: Bacterial genera in groundwater samples and enrichment cultures identified by 16S rRNA amplicon sequencing on an Illumina MiSeq platform. The taxonomic assignment of 16S rRNA sequences to bacterial genera was done with QIIME. The abundance of bacterial genera is expressed as percentage of total 16S rRNA sequences and as absolute number of sequence counts. Bacterial genera involved in oxygen-independent or low-oxygen-dependent aromatic compound degradation were printed in bold and marked with an asterisk.

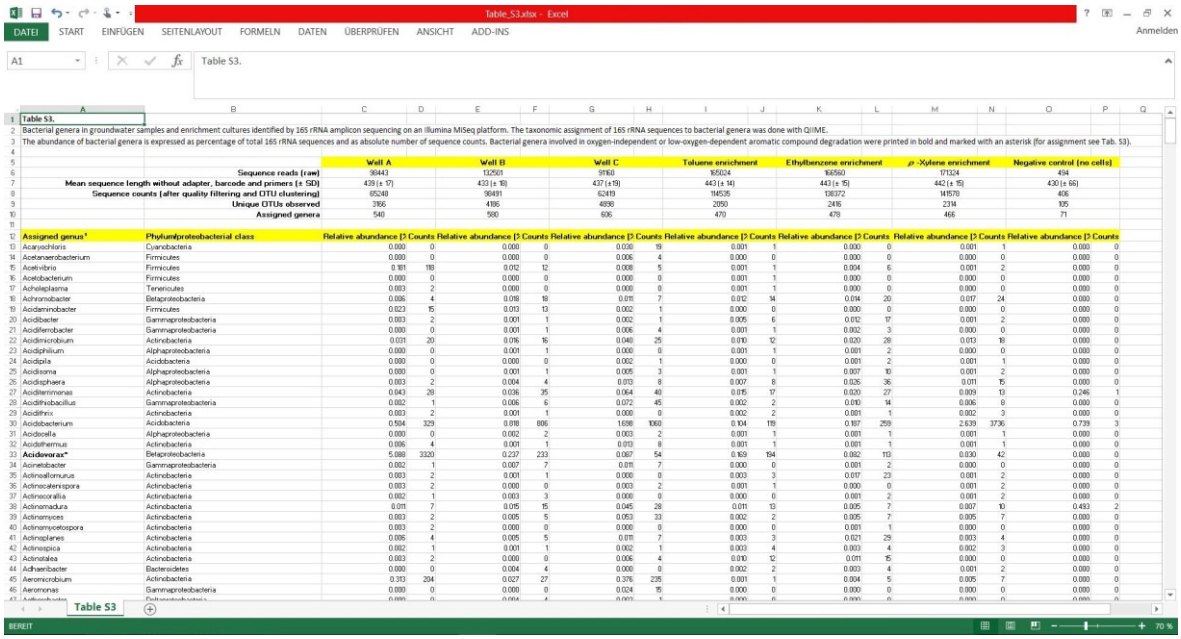


Table S3.

Bacterial genera in groundwater samples and enrichment cultures identified by 16S rRNA amplicon sequencing on an Illumina MiSeq platform. The taxonomic assignment of 16S rRNA sequences to bacterial genera was done with QIIME.
The abundance of bacterial genera is expressed as percentage of total 16S rRNA sequences and as absolute number of sequence counts. Bacterial genera involved in oxygen-independent or low-oxygen-dependent aromatic compound degradation were printed in bold and marked with an asterisk (for assignment see Tab. S3).

	Well A	Well B	Well C	Toluene enrichment	Ethylbenzene enrichment	*p* -Xylene enrichment	Negative control (no cells)
Sequence reads (raw)	38443	112501	91160	105024	116560	171124	494
Mean sequence length without adapter, barcode and primers (± SD)	439 (± 17)	433 (± 18)	437 (±19)	443 (± 14)	443 (± 15)	442 (± 15)	430 (± 66)
Sequence counts (after quality filtering and OTU clustering)	65248	90491	62419	114535	138372	141570	406
Unique OTUs observed	3166	4186	4898	2050	2416	2314	105
Assigned genera	540	560	606	470	478	468	71

Assigned genus*	Phylum/proteobacterial class	Relative abundance [%]	Counts	Relative abundance [%]	Counts	Relative abundance [%]	Counts	Relative abundance [%]	Counts	Relative abundance [%]	Counts	Relative abundance [%]	Counts	Relative abundance [%]	Counts
Asaryachloris	Cyanobacteria	0.000	0	0.000	0	0.030	19	0.001	1	0.000	0	0.001	1	0.000	0
Acetanaerobacterium	Firmicutes	0.000	0	0.000	0	0.006	4	0.000	0	0.000	0	0.000	0	0.000	0
Acetivibrio	Firmicutes	0.181	118	0.012	12	0.008	5	0.001	1	0.004	6	0.001	2	0.000	0
Acetobacterium	Firmicutes	0.000	0	0.000	0	0.000	0	0.001	1	0.000	0	0.000	0	0.000	0
Achaleplasma	Tenericutes	0.003	2	0.000	0	0.000	0	0.001	1	0.000	0	0.000	0	0.000	0
Achromobacter	Betaproteobacteria	0.006	4	0.018	18	0.011	7	0.012	14	0.014	20	0.017	24	0.000	0
Acidaminobacter	Firmicutes	0.023	15	0.013	13	0.002	1	0.000	0	0.000	0	0.000	0	0.000	0
Acidibacter	Gammaproteobacteria	0.003	2	0.001	1	0.002	1	0.005	6	0.012	17	0.001	2	0.000	0
Acidiferrobacter	Gammaproteobacteria	0.000	0	0.001	1	0.006	4	0.001	1	0.002	3	0.000	0	0.000	0
Acidimicrobium	Actinobacteria	0.031	20	0.016	16	0.040	25	0.010	12	0.020	28	0.013	18	0.000	0
Acidiphilium	Alphaproteobacteria	0.000	0	0.001	1	0.000	0	0.001	1	0.001	2	0.000	0	0.000	0
Acidipila	Acidobacteria	0.000	0	0.000	0	0.002	1	0.000	0	0.001	2	0.001	1	0.000	0
Acidisoma	Alphaproteobacteria	0.000	0	0.001	1	0.005	3	0.001	1	0.007	10	0.001	2	0.000	0
Acidisphaera	Alphaproteobacteria	0.003	2	0.004	4	0.013	8	0.007	8	0.025	36	0.011	15	0.000	0
Aciditerrimonas	Actinobacteria	0.043	28	0.036	35	0.064	40	0.015	17	0.020	27	0.009	13	0.246	1
Acidithiobacillus	Gammaproteobacteria	0.002	1	0.006	6	0.072	45	0.002	2	0.010	14	0.006	9	0.000	0
Acidithrix	Actinobacteria	0.003	2	0.001	1	0.000	0	0.002	2	0.001	1	0.002	3	0.000	0
Acidobacterium	Acidobacteria	0.504	329	0.918	806	1.598	1060	0.104	119	0.187	259	2.639	3736	0.739	3
Acidocella	Alphaproteobacteria	0.000	0	0.002	2	0.003	2	0.001	1	0.001	1	0.001	1	0.000	0
Acidothermus	Actinobacteria	0.006	4	0.001	1	0.013	8	0.001	1	0.001	1	0.001	1	0.000	0
Acidovorax*	Betaproteobacteria	5.088	3320	0.237	233	0.067	54	0.169	194	0.092	153	0.030	42	0.000	0
Acinetobacter	Gammaproteobacteria	0.002	1	0.007	7	0.011	7	0.000	0	0.001	2	0.000	0	0.000	0
Actinoallomurus	Actinobacteria	0.003	2	0.001	1	0.000	0	0.003	3	0.017	23	0.001	2	0.000	0
Actinocatenispora	Actinobacteria	0.003	2	0.000	0	0.003	2	0.001	1	0.000	0	0.001	2	0.000	0
Actinocorallia	Actinobacteria	0.002	1	0.003	3	0.000	0	0.000	0	0.001	2	0.001	2	0.000	0
Actinomadura	Actinobacteria	0.011	7	0.015	15	0.045	28	0.011	13	0.005	7	0.007	10	0.493	2
Actinomyces	Actinobacteria	0.003	2	0.005	5	0.053	33	0.002	2	0.005	7	0.005	7	0.000	0
Actinomycetospora	Actinobacteria	0.003	2	0.000	0	0.000	0	0.000	0	0.001	1	0.000	0	0.000	0
Actinoplanes	Actinobacteria	0.006	4	0.005	5	0.011	7	0.003	3	0.021	29	0.003	4	0.000	0
Actinospica	Actinobacteria	0.002	1	0.001	1	0.002	1	0.003	4	0.003	4	0.002	3	0.000	0
Actinotalea	Actinobacteria	0.003	2	0.000	0	0.006	4	0.010	12	0.011	15	0.000	0	0.000	0
Adhaeribacter	Bacteroidetes	0.000	0	0.004	4	0.000	0	0.002	2	0.003	4	0.001	2	0.000	0
Aeromicrobium	Actinobacteria	0.313	204	0.027	27	0.376	235	0.001	1	0.004	5	0.005	7	0.000	0
Aeromonas	Gammaproteobacteria	0.000	0	0.000	0	0.024	15	0.000	0	0.000	0	0.000	0	0.000	0

For full table, see supplemental CD-ROM (./Manuscript_I/Table_S3.xlsx) or download from: https://ars.els-cdn.com/content/image/1-s2.0-S0043135417310357-mmc3.xlsx

Table S4: Bacterial genera that were assigned as aromatic compound-degrading bacteria (ACDB) in oxygen-depleted environments based on published results commented in the column 'comments and references'. Most of the genera listed possess the anaerobic benzoyl-CoA pathway (oxygen-independent ACDB, short: no-ox ACDB) and/or the box pathway (low-oxygen-dependent ACDB, short: low-ox ACDB) for aromatic compound degradation.

Genus	no-ox ACDB	low-ox ACDB	box pathway [1]	Comments and references
Acidovorax		X	X	Presence of oxygen-independent benzoyl-CoA reductase in a denitrifying, aromatic compound- degrading isolate (Song & Ward, 2005); enrichment of *Acidovorax* spp. in microcosms amended with benzene and nitrate (Fahy *et al*., 2006)
Afipia		X	X	Phenyl acetate assimilation and nitrate reduction observed in *Afipia* spp. (La Scola *et al*., 2002); genes for benzoate transport and degradation present in the genome of *A. birgiae* 32632^{T} (Pagnier *et al*., 2012)
Azoarcus	X		X	Boll *et al.* (2014) and references within
Blastochloris	X			Phototrophic, anoxic utilization of toluene by *Blastochloris sulfoviridis* (Zengler *et al*., 1999)
Bradyrhizobium		X	X	Presence of oxygen-independent benzoyl-CoA reductase in denitrifying, aromatic compound-degrading isolates (Song & Ward, 2005); genes for nitrate reduction and degradation of benzoate via benzoyl-CoA present in the genome of *B. japonicum* CPAC 15 (Godoy *et al*., 2008)
Burkholderia		X	X	Growth of *Burkolderia xenovorans* with benzoate and biphenyl; nitrate reduction strain-dependent (Goris *et al*., 2004)
Cupriavidus		X	X	*Cupriavidus necator* JMP134 uses at least 60 different aromatic compounds as sole carbon and energy source; *C. necator* possess almost all main oxygen-dependent, ring-cleaving pathways, and encodes gene clusters for denitrification (Lykidis *et al*., 2010)
Curvibacter		X	X	*Curvibacter* is microaerobic (Ding & Yokota, 2004); denitrification was observed in isolated strains (Nishizawa *et al*., 2013); aromatic compound degradation was not investigated so far
Dechloromonas [2]		X		*Dechloromonas* strains RCB and JJ were reported to degrade benzene in the absence of oxygen under nitrate-reducing conditions (Coates *et al*., 2001), however genes coding for oxygen-independent aromatic compound degradation pathways are absent in the genome of *D. aromatica* strain RCB (Salinero *et al*., 2009); the release of oxygen during denitrification is under debate (Weelink *et al*., 2010)
Delftia		X	X	Anaerobic growth of *Delftia* spp. using electrodes as electron acceptors (Jangir *et al*., 2016); *Delftia acidovorans* Cs1-4, isolated from coal tar contaminated soil, encodes oxygen-dependent aromatic compound degradation pathways (Shetty *et al*., 2015)
Denitratisoma	X			Highly enriched *Denitratisoma* sp. degrades *p*-xylene in the presence of nitrate via fumarate addition (Rotaru *et al*., 2010)
Desulfatiglans	X			Boll *et al.* (2014) and references within; reclassification of *Desulfobacterium anilini* as *Desulfatiglans anilini* (Suzuki *et al*., 2014)
Desulfatirhabdium	X			*Desulfatirhabdium butyrativroans* degrades aromatic compounds in the presence of sulfate (Balk *et al*., 2008); ring-cleaving hydrolase (accession-no.: WP_028323760) and benzoyl-CoA reductase (subunit B: WP_028324510) encoded in the genome
Desulfitobacterium	X			Boll *et al.* (2014) and references within
Desulfobacterium	X			Boll *et al.* (2014) and references within
Desulfobacula	X			Boll *et al.* (2014) and references within
Desulfocapsa	X			Degradation of toluene under sulfidogenic conditions by *Desulfobulbaceae* bacterium Tol-SR; according to Abu Laban *et al.* (2015a) this strain has the highest similarity to *Desulfocapsa* and was therefore assigned to this genus
Desulfococcus	X			Boll *et al.* (2014) and references within
Desulfomonile	X			Boll *et al.* (2014) and references within
Desulfonema	X			Boll *et al.* (2014) and references within
Desulfosarcina	X			Boll *et al.* (2014) and references within
Desulfosporosinus	X			Genome of uncultivated *Desulfosporosinus* sp. Strain Tol-M contains genes for oxygen-independent aromatic compound degradation (Abu Laban *et al*., 2015b)
Desulfotignum	X			Boll *et al.* (2014) and references within
Desulfotomaculum	X			Boll *et al.* (2014) and references within

Table S4: (continued)

Genus	no-ox ACDB	low-ox ACDB	box pathway [1]	Comments and references
Geobacter	X			Boll *et al.* (2014) and references within
Geopsychrobacter	X			Boll *et al.* (2014) and references within
Georgfuchsia	X			Boll *et al.* (2014) and references within
Herminiimonas	X		X	Degradation of toluene in the presence of nitrate by *Herminiimonas* sp. CN (uncultivated bacterium); gene clusters for oxygen-independent and low-oxygen-dependent degradation of aromatic compounds via benzoyl-CoA are present (Kim *et al.*, 2014)
Hydrogenophaga		X	X	Nitrate reduction and hydroxybenzoate assimilation observed (Kim *et al.*, 2010)
Magnetospirillum	X		X	Boll *et al.* (2014) and references within
Methylibium		X	X	Nitrate reduction and assimilation of various aromatic compounds by *Methylibium petroleiphilum* (Nakatsu *et al.*, 2006)
Nevskia		X	X	Growth with benzoate, anaerobic growth was not observed (Stürmeyer *et al.*, 1998)
Novosphingobium		X	X	Gene cluster for aromatic compound degradation present in the genome of *Novosphingobium lindaniclasticum* LE124 (Saxena *et al.*, 2013); members of the genus commonly reduce nitrate (Takeuchi *et al.*, 2001)
Pelotomaculum	X			Boll *et al.* (2014) and references within
Polaromonas		X	X	Growth with aromatic compounds; gene clusters for aromatic compound degradation and genes for denitrification present in the genome of *Polaromonas naphthalenivorans* CJ2 (Yagi *et al.*, 2009)
Pseudomonas[2]		X		Presence of oxygen-independent benzoyl-CoA reductase in denitrifying, aromatic compound- degrading isolate (Song & Ward, 2005); naphthalene degradation observed in denitrifying isolate (Rockne *et al.*, 2000)
Ramlibacter	X		X	Presence of ring-cleaving hydrolase (accession-no.: WP_061497079) and benzoyl-CoA reductase (subunit B: AMO22489) in the genome of *Ramlibacter tataouinensis*; aromatic compound degradation was not investigated so far
Rhodoferax		X	X	Degradation of benzoate in the presence of Fe(III) by *Rhodoferax ferrireducens* (Finneran *et al.*, 2003); genes for box pathway encoded in the corresponding genome (Risso *et al.*, 2009)
Rhodomicrobium	X			Boll *et al.* (2014) and references within
Rhodopseudomonas	X		X	Boll *et al.* (2014) and references within
Rhodovulum	X			Boll *et al.* (2014) and references within
Sedimenticola	X			Boll *et al.* (2014) and references within
Sporotomaculum	X			Boll *et al.* (2014) and references within
Sulfuritalea	X		X	Anaerobic growth of *Sulfuritalea hydrogenivorans* sk43H with benzoate and nitrate (Kojima & Fukui, 2011); benzoyl-CoA reductase (accession no.: BAO29438-BAO29441) and ring-cleaving hydrolase (BAO29449) encoded in the genome
Syntrophorhabdus	X			Boll *et al.* (2014) and references within
Syntrophus	X			Boll *et al.* (2014) and references within
Thauera	X		X	Boll *et al.* (2014) and references within
Variovorax		X	X	Members of *Variovorax* are facultative anaerobes; they occur in polluted habitats and can degrade aromatic compounds (Satola *et al.*, 2013)
Xenophilus		X	X	Utilization of hydroxybenzoates by *Xenophilus azovorans* (Blümel *et al.*, 2001); anaerobic growth was not observed
Xylophilus		X	X	Reduction of nitrate by isolates of *Xylophilus* (Dreo *et al.*, 2005), aromatic compound degradation was not investigated so far

[1] The key enzymes of the box pathway, benzoyl-CoA 2,3-epoxidase (BoxB) and 2,3-epoxybenzoyl-CoA dihydrolase (BoxC), were identified by a BLAST search using BoxB (accession no. Q9AIX7) and BoxC (accession no. Q84HH6) of *Azoarcus evansii* as template (previously described by Rather *et al.*, 2010). Some no-ox ACDB also harbor genes encoding key enzymes of the box pathway.

[2] None of the two pathways for aromatic compound degradation could be assigned to the genera *Dechloromonas* and *Pseudomonas*.

Table S5: Accession numbers and CDS regions of *bamA* reference sequences[1].

Reference organism	Protein accession no.	CDS region in nucleotide
Azoarcus sp. EbN1	WP_011238812	NC_006513.1: 3146722-3147852
Azoarcus evansii	CAD21638	AJ428529.1: 11281-12408
Azoarcus sp. CIB	WP_050415414	NZ_CP011072.1: 1821072-1822202
Azoarcus sp. KH32C	WP_015435765	NC_020516.1: 2426814-2427944
Clostridia bacterium enrichment culture BF	ADJ93959	GU357949.1: 1-1128
Clostridiales bacterium PH28	KKM12491	LAKY01000013.1: c3370-2240
Desulfatiglans anilini	ACZ58169	GU057387.1: 1-710
Desulfatirhabdium butyrativorans DSM 18734	WP_028323760	NZ_AUCU01000013.1: 120261-121394
Desulfobacula toluolica Tol2	WP_014955908	NC_018645.1: c462643-461507
Desulfobulbaceae bacterium Tol-SR	KGO35810	JROS01000001.1: c45613-44480
Desulfococcus multivorans	WP_020875289	NZ_CP015381.1: 2803037-2804170
Desulfomonile tiedjei DSM 6799	WP_014809339	NC_018025.1: 1747556-1748686
Desulfosarcina cetonica	ACZ58170	GU057388.1: 1-704
Desulfosarcina ovata	ACZ58171	GU057389.1: 1-704
Desulfotignum phosphitoxidans DSM 13687	WP_006965674	NZ_APJX01000003.1: 479221-480357
Desulfotomaculum gibsoniae DSM 7213	WP_006522593	NC_021184.1: 2007228-2008358
Desulfotomaculum sp. BICA1-6	KJS78616	JUED01000008.1: 10915-12045
Geobacter bemidjiensis Bem	WP_012529874	NC_011146.1: 1666047-1667186
Geobacter metallireducens GS-15	WP_004512529	NC_007517.1: c3720299-3719175
Geobacter pickeringii G13	WP_039742573	NZ_CP009788.1: c2108882-2107740
Geobacter sp. M21	WP_015838068	NC_012918.1: c3271808-3270663
Georgfuchsia toluolica G5G6[2]	CBH32498	FN565188.1: 1-267
Herminiimonas sp. CN	WP_025918185	NZ_AVCC01000092.1: 4424-5554

Table S5: (*continued)*

Reference organism	Protein accession no.	CDS region in nucleotide
Magnetospirillum magneticum AMB-1	WP_011384542	NC_007626.1: c2320470-2319337
Magnetospirillum sp. pMbN1	AIW63112	KF941542.1: 1-1131
Magnetospirillum sp. XM-1	WP_068434212	NZ_LN997848.1: c2893014-2891881
Peptococcaceae bacterium BRH_c8a	KJS12707	LADP01000019.1: c121223-120093
Rhodomicrobium udaipurense JA643	WP_037240367	NZ_JFZJ01000116.1: c8746-7610
Rhodomicrobium vannielii ATC 17100	WP_013417732	NC_014664.1: 40627-41763
Rhodovulum sp. PH10	WP_008387066	NZ_AKZI01000060.1: 29200-30348
Sulfuritalea hydrogenivorans sk43H	WP_041098456	NZ_AP012547.1: 1681125-1682255
Syntrophorhabdus aromaticivorans UI	WP_028894052	NZ_KI867150.1: 932365-933510
Syntrophus aciditrophicus SB	WP_011418920	NC_007759.1: 3032541-3033689
Thauera aromatica	CAA12245.1	AJ224959.2: 4455-5588
Thauera chlorobenzoica	WP_075148408	NZ_CP018839.1: 2319817-2320950
Thauera sp. MZ1T	WP_004303024	NC_011662.2: c3195485-3194352

[1] The *bamA* reference list includes sequences from well characterized pure cultures, enrichment cultures with sequenced (meta)genomes as well as sequences of microorganisms that matched the newly retrieved *bamA* sequences with highest score in a BLASTn search (nucleotide collection (nr/nt) database, excluding uncultured/environmental samples; Altschul *et al.*, 1990).

[2] For *Georgfuchsia toluolica* G5G6 only a partial *bamA* sequence is publicly available. The full *bamA* sequence used for alignment was provided by courtesy of M. J. Oosterkamp (Delft University of Technology) and A. J. M. Stams (Wageningen University & Research).

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Table S1: *Sulfuritalea*-related *bamA* sequences.

Designation	GenBank number	Sampling site	Nucleotide Identity [a] (%)	Comment	Reference
clone bamA_seq90	FN564179.1	landfill leachate plume	94	Figure 4 in reference, *bamA* sequences were assigned to *Georgfuchsia*/*Azoarcus* clade	Staats *et al*. (2011)
clone 0c_1999(4C)	FN564320.1	landfill leachate plume	93	Figure 4 in reference, *bamA* sequences were assigned to *Georgfuchsia*/*Azoarcus* clade	Staats *et al*. (2011)
clone Hansemann-5	GU057404.1	former benzene production plant	93	Figure 2 in reference, BPP *bamA* clones (*bamA* PCR assay)	Kuntze *et al*. (2011)
clone Hansemann-6	GU057405.1	former benzene production plant	91	Figure 2 in reference, BPP *bamA* clones (*bamA* PCR assay)	Kuntze *et al*.(2011)
RIFCSPLOWO2_ 02_FULL_63_24	MHZX01000067.1	suboxic/anoxic aquifer adjacent to the Colorado River	93	bamA sequence of a Rhodocyclales bacterium identified by whole genome shotgun sequencing	Anantharaman *et al*. (2016)
bamA-OTU12	-	diesel fuel contaminated Antarctic soil	(91) [c]	Table 2 in reference, *bamA*-OTUs derived from *bamA* sequences of *bamA* clone libraries	Sampaio *et al*.(2017)
bamA-OTU19	-	diesel fuel contaminated Antarctic soil	(92) [c]	Table 2 in reference, *bamA*-OTUs derived from *bamA* sequences of *bamA* clone libraries	Sampaio *et al*. (2017)
clone GW-A-21 clone GW-C-22 clone GW-B-02	LT800524.1 LT800648.1 LT800567.1	coal-tar polluted groundwater	94	Figure 3 in reference, *bamA* clones were assigned to clade I (*Azoarcus*/*Sulfuritalea*/*Georgfuchsia*-related)	Sperfeld *et al*.(2018) [b]

[a] The nucleotide sequence identity was calculated for partial *bamA* sequences (position 200 to 500 bp; base pair coordinates for *bamA* gene *SUTH_01658* of *S. hydrogenivorans* sk43H)

[b] Three representatives with highest nucleotide sequence identity were stated in the table. In the study of Sperfeld *et al.* (2018), 27 clones were identified with nucleotide sequence identity values of 91-94%.

[c] The number in parenthesis indicates the protein identity.

Table S2: Genes of *Sulfuritalea hydrogenivorans* sk43H associated with anaerobic aromatic compound degradation, the predicted function of the gene products and nucleotide sequence identity to the closest match in "*Aromatoleum aromaticum*" strain EbN1. Genome comparison was done using the sequence based comparison tool implemented in SEED Viewer (Overbeek *et al.*, 2005).

Locus tag *S. hydrogenivorans* sk43H	Closest match "*A. aromaticum*" strain EbN1	Nucleotide identity (%)	Gene name	Predicted function
Anaerobic degradation of benzoate and upper benzoyl-CoA pathway (*bcr/bzd* gene cluster)				
SUTH_01645	*ebA5282*	87.9	*bcrC*	Benzoyl-CoA reductase (gamma subunit)
SUTH_01646	*ebA5284*	87.7	*bcrB*	Benzoyl-CoA reductase (beta subunit)
SUTH_01647	*ebA5286*	77.7	*bcrD*	Benzoyl-CoA reductase (delta subunit)
SUTH_01648	*ebA5287*	90.4	*bcrA*	Benzoyl-CoA reductase (alpha subunit)
SUTH_01649	*ebA5289*	85.1	*fxd*	Ferredoxin
SUTH_01650	*ebB187*	55.5		Conserved hypothetical protein
SUTH_01651	*ebA5292*	70.5		Conserved regulatory protein
SUTH_01652	*ebA5293*	76.4		Conserved hypothetical protein
SUTH_01653	*ebA5294*	68.0	*bzdV*	NADPH:ferredoxin oxidoreductase
SUTH_01654	*ebA5296*	87.1	*dch*	Dienoyl-CoA hydratase
SUTH_01655	*ebA5297*	79.4	*had*	6-Hydroxycyclohex-1-ene-1-carboxyl-CoA dehydrogenase
SUTH_01656	*ebA5298*	78.9	*oah*	6-Oxo-cyclohex-1-ene-carbonyl-CoA hydrolase
SUTH_01657	*ebA5300*	86.7	*bzdZ*	Dehydrogenase
SUTH_01658	*ebA2993*	86.2	*gcdH*	Glutaryl-CoA dehydrogenase
SUTH_01659	*ebA5301*	68.1	*bclA*	Benzoate-CoA ligase
SUTH_01660	*ebA5303*	69.5		Benzoate ABC transporter (extracellular ligand-binding receptor)
SUTH_01661	*ebA5304*	71.1		Putative benzoate ABC transporter
SUTH_01662	*ebA5306*	61.2		Putative benzoate ABC transporter
SUTH_01663	*ebA5307*	65.1		Putative benzoate ABC transporter
SUTH_01664	*ebA5309*	72.9		Putative benzoate ABC transporter
SUTH_01665	*ebA2753*	61.9		Alpha/beta hydrolase
SUTH_01666	*ebA2036*	49.6		Putative thioesterase
Aerobic benzoate hybrid pathway (*box* gene cluster)				
SUTH_01641	*ebA2766*	63.0	*boxA*	Benzoyl-CoA oxygenase component A (reductase)
SUTH_01642	*ebA2765*	72.9	*boxB*	Benzoyl-CoA oxygenase component B (monooxygenase)
SUTH_01643	*ebA2763*	71.9	*boxC*	Benzoyl-CoA dihydrodiol lyase
SUTH_01644	*ebA2762*	58.6	*boxR*	Transcriptional regulator
Anaerobic peripheral pathway of phenylacetate (*pad* gene cluster)				
SUTH_01618	*ebB192*	81.1		Thioesterase
SUTH_01619	*ebA5403*	75.6		4-Hydroxyphenylacetate-CoA ligase
SUTH_01620	*ebA5390*	74.4	*padR*	Regulator of anaerobic phenylacetate metabolism
SUTH_01621	*ebA5392*	65.2	*padA*	Chaperon for phenylacetyl-CoA:acceptor oxidoreductase
SUTH_01622	*ebA5393*	75.3	*padB*	Phenylacetyl-CoA:acceptor oxidoreductase (large subunit)
SUTH_01623	*ebA5395*	81.8	*padC*	Phenylacetyl-CoA:acceptor oxidoreductase (medium subunit)
SUTH_01624	*ebA5396*	55.9	*padD*	Phenylacetyl-CoA:acceptor oxidoreductase (membrane anchor)
SUTH_01625	*ebA5397*	80.0	*padE*	Phenylglyoxylate:acceptor oxidoreductase (gamma subunit)
SUTH_01626	*ebB191*	88.8	*padF*	Phenylglyoxylate:acceptor oxidoreductase (delta subunit)
SUTH_01627	*ebA5399*	83.7	*padG*	Phenylglyoxylate:acceptor oxidoreductase (alpha subunit)
SUTH_01628	*ebA5400*	73.9	*padH*	Phenylglyoxylate:acceptor oxidoreductase (epsilon subunit)
SUTH_01629	*ebA5401*	80.8	*padI*	Phenylglyoxylate:acceptor oxidoreductase (beta subunit)
SUTH_01630	*ebA5402*	75.8	*padJ*	Phenylacetate-CoA ligase
SUTH_01631	*ebA5362*	55.2	*dctP*	TRAP transporter (solute receptor)
SUTH_01632	*ebB188*	46.5	*dctQ*	TRAP transporter (small permease)
SUTH_01633	*ebA5367*	69.1	*dctM*	TRAP transporter (large permease)
SUTH_01634	-	-		Hypothetical protein
Anaerobic peripheral pathway of cinnamate (*cou* gene cluster)				
SUTH_01227	*ebA3558*	62.9		Hydrophobic amino acid uptake transporter
SUTH_01228	*ebA3559*	64.2		Hydrophobic amino acid uptake transporter
SUTH_01229	*ebA3560*	66.7		Hydrophobic amino acid uptake transporter
SUTH_01230	*ebA5316*	53.2		Periplasmic 3-phenylpropanoid binding protein
SUTH_01231	*ebA5317*	65.2		3-Phenylpropanoid-CoA ligase
SUTH_01232	*ebA5318*	75.7		Enoyl-CoA hydratase/isomerase
SUTH_01233	-	-		TIM-barrel fold metal-dependent hydrolase
SUTH_01234	*ebA5321*	70.1		Acyl-CoA dehydrogenase

Table S3: Genes coding for enzymes putatively involved in β-oxidation reactions of the lower benzoyl-CoA pathway in *Sulfuritalea hydrogenivorans* sk43H and nucleotide sequence identity to the closest match in "*Aromatoleum aromaticum*" strain EbN1. The table was adapted from Rabus *et al.* (2005).

Locus tag *S. hydrogenivorans* sk43H	Closest match "*A. aromaticum*" strain EbN1	Nucleotide identity (%)
CoA ligases, AMP forming		
SUTH_02442	*ebA7220*	78.7
SUTH_01630	*ebA5402*	75.8
SUTH_02307	*ebA172*	76.5
SUTH_02599	*ebA4749*	69.7
SUTH_01659	*ebA5301*	68.1
SUTH_01492	*ebA4666*	67.1
SUTH_01231	*ebA5317*	65.2
SUTH_01723	*ebA5700*	57.4
SUTH_00168	*ebA5760*	48.7
SUTH_00999	*ebA4326*	31.3
CoA ligases, AMP forming		
SUTH_02802	*ebA1271*	93.6
SUTH_02803	*ebA1272*	88.1
SUTH_01619	*ebA5403*	75.6
SUTH_02470	*ebA6829*	65.4
Sirtuin-type regulators for CoA ligases		
SUTH_02147	*ebA3329*	35.7
CoA-transferases, Family III		
SUTH_03237	*ebA2992*	69.7
SUTH_00632	*ebA3792*	61.4
SUTH_02117	*ebA5640*	35.7
Acyl-CoA dehydrogenases		
SUTH_01658	*ebA2993*	86.2
SUTH_00533	*ebA4656*	81.3
SUTH_00539	*ebA3640*	80.0
SUTH_01526	*ebA736*	70.8
SUTH_01234	*ebA5321*	70.1
SUTH_01488	*ebA6506*	67.8
SUTH_02338	*ebA5668*	55.1
SUTH_00088	*ebA1985*	50.7
SUTH_00089	*ebA5641*	49.1
SUTH_02320	*ebA5669*	48.7
Electron transfer flavoproteins		
SUTH_01486	*ebA6511*	83.5
SUTH_01487	*ebA6510*	76.2
SUTH_01490	*c1A56*	75.3
Enoyl-CoA hydratases (S)-specific		
SUTH_01654	*ebA5296*	87.1
SUTH_03028	*ebA1321*	77.7
SUTH_01232	*ebA5318*	75.7
SUTH_01722	*ebA6516*	72.9
SUTH_01643	*ebA2763*	71.9
SUTH_03477	*ebA5836*	68.7
SUTH_03559	*ebA3691*	64.6
SUTH_01525	*ebA722*	62.6
SUTH_02808	*ebA3542*	62.0
SUTH_00544	*ebA3642*	51.5
SUTH_02073	*ebA2313*	47.4

Table S3: Continued.

Locus tag *S. hydrogenivorans* sk43H	Closest match "*A. aromaticum*" strain EbN1	Nucleotide identity (%)
(R)-specific MaoC-like enoyl-CoA hydratases		
SUTH_02296	*ebA4434*	59.6
Thiolases without small subunits		
SUTH_00537	*ebA3639*	80.4
SUTH_02810	*ebA5729*	77.8
SUTH_01122	*ebA5202*	74.0
SUTH_00071	*c1A212*	71.4
SUTH_01484	*ebA2314*	61.4
Short chain alcohol dehydrogenases		
SUTH_01657	*ebA5300*	86.7
SUTH_02644	*ebA4729*	86.6
SUTH_02200	*ebA5457*	77.1
SUTH_00835	*ebA5126*	75.2
SUTH_02643	*ebA4731*	71.5
SUTH_00188	*ebA3368*	67.9
SUTH_00623	*ebA4742*	68.8
SUTH_02651	*c2A173*	60.8
SUTH_01483	*ebA2315*	56.1
SUTH_01134	*ebA5620*	50.1
SUTH_02529	*ebA4458*	31.3
Other alcohol dehydrogenases		
SUTH_03212	*ebA4396*	77.8
SUTH_02443	*ebA7223*	62.4
SUTH_02609	*ebA4623*	40.5
SUTH_01396	*ebA5761*	32.7
Aldehyde dehydrogenases		
SUTH_03233	*ebA4380*	75.5
SUTH_03121	*ebA935*	72.8
SUTH_01299	*ebA4761*	71.6
SUTH_01736	*ebA2996*	71.1
SUTH_01118	*ebA510*	64.0
SUTH_02341	*ebA5713*	37.5

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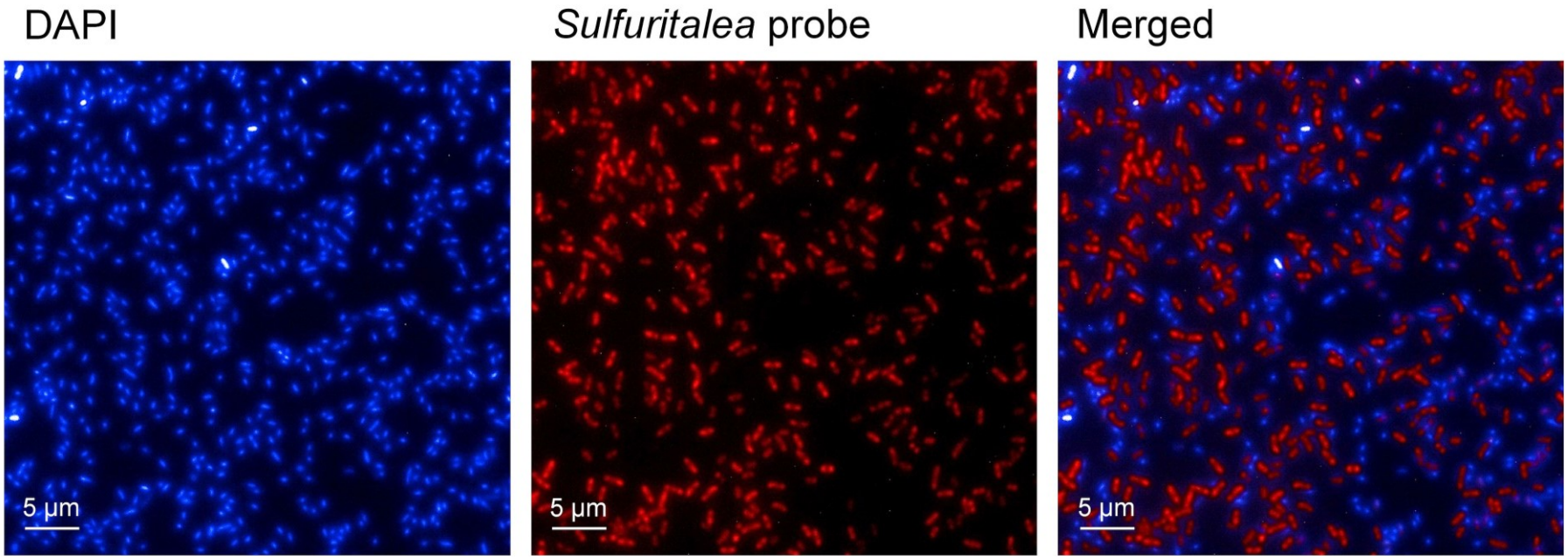


Figure S1: Visual detection of *Sulfuritalea*-related microorganisms in culture pMB18 grown with *p*-methylbenzoate by applying FISH.

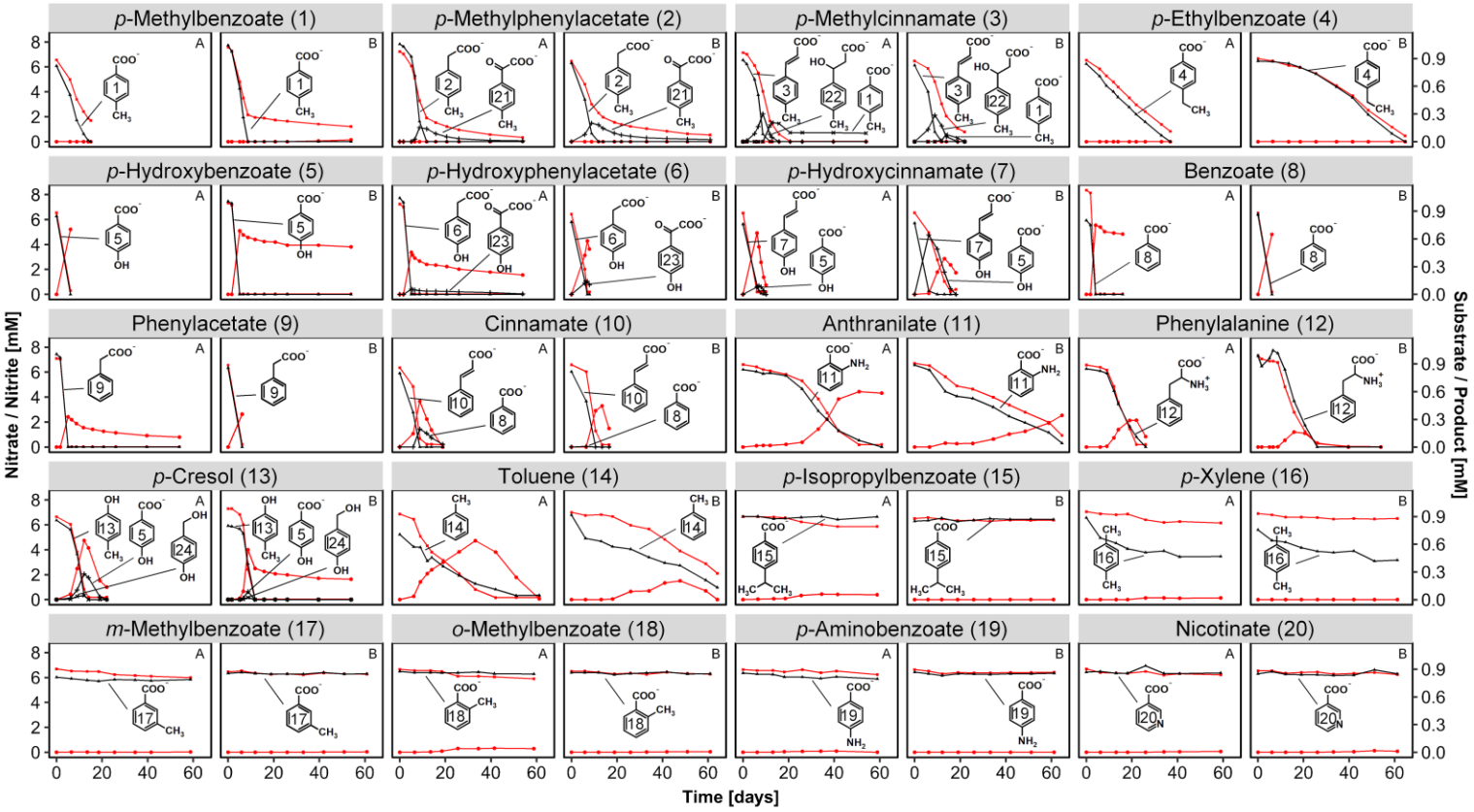


Figure S2.Consumption of aromatic substrates by culture pMB18. The filled triangle (▲) indicates the aromatic substrate, the red filled square (■) nitrate and the red filled circle (●) nitrite. Intermediates are plotted as (+) and (x). For each substrate, two cultures are depicted. (1) *p*-methylbenzoate; (2) *p*-methylphenylacetate; (3) *p*-methylcinnamate; (4) *p*-ethylbenzoate; (5) *p*-hydroxybenzoate; (6) *p*-hydroxyphenylacetate; (7) *p*-hydroxycinnamate; (8) benzoate; (9) phenylacetate; (10) cinnamate; (11) anthranilate; (12) phenylalanine; (13) *p*-cresol; (14) toluene; (15) *p*-isopropylbenzoate; (16) *p*-xylene; (17) *m*-methylbenzoate; (18) *o*-methylbenzoate; (19) *p*-aminobenzoate; (20) nicotinate; (21) *p*-methylphenylglyoxylate; (22) 3-Hydroxy-3-(4-methylphenyl)propanate; (23) *p*-hydroxyphenylglyoxylate; (24) *p*-hydroxybenzyl alcohol.

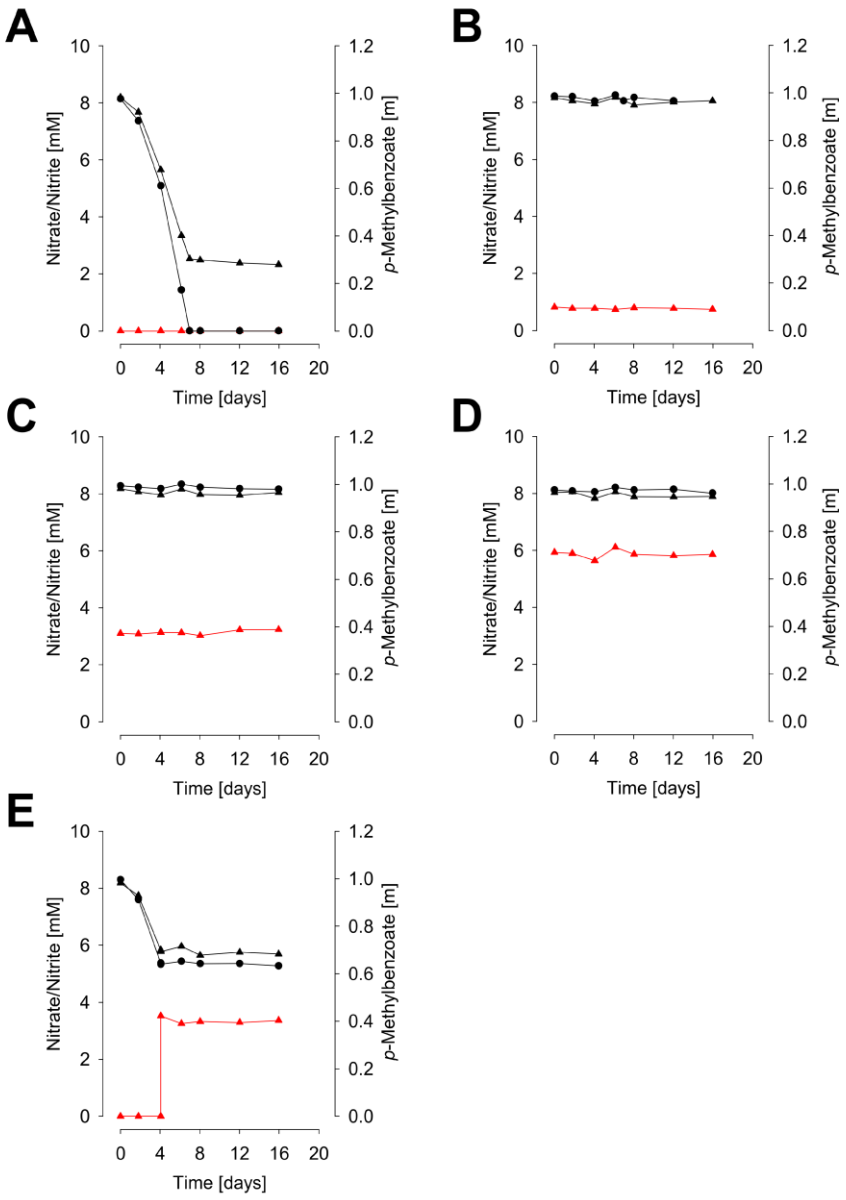
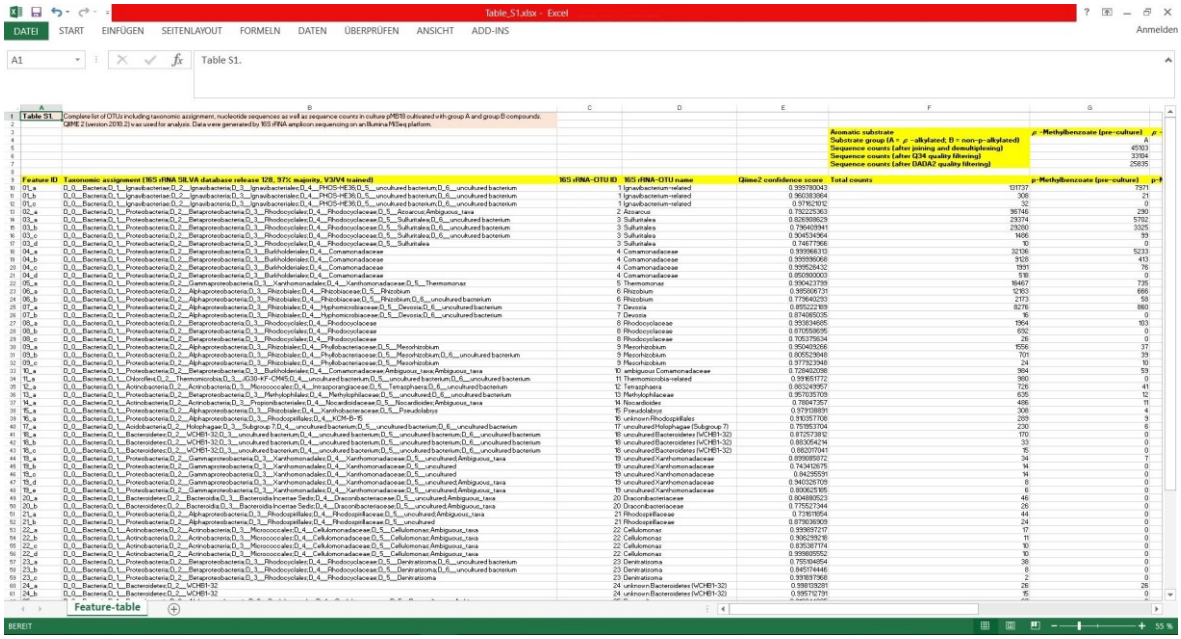


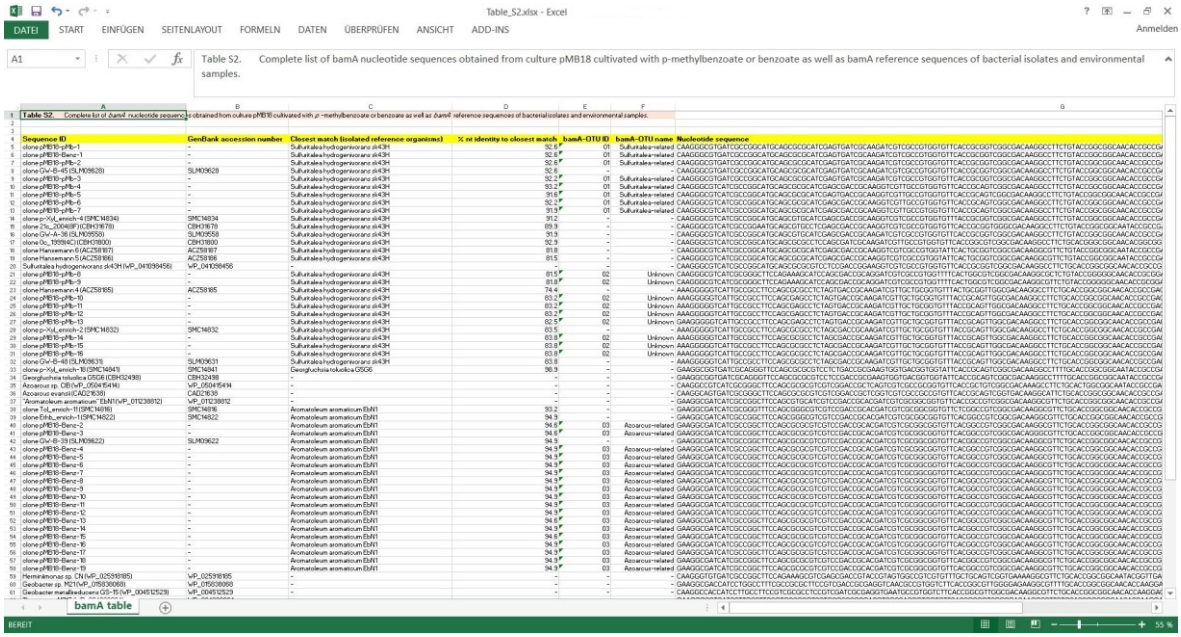
Figure S3: Consumption of *p*-methylbenzoate by culture pMB18 in the absence (**A**) or presence of (**B**) 1 mM, (**C**) 3 mM, and (**D**) 6 mM nitrite. (**E**) Nitrite addition (3 mM) during exponential growth of culture pMB18. The filled circle (●) indicates *p*-methylbenzoate, the filled triangle (▲) nitrate and the red filled triangle (▲) nitrite.

Table S1: Complete list of OTUs including taxonomic assignment, nucleotide sequences as well as sequence counts in culture pMB18 cultivated with group A and group B compounds. Data were generated by 16S rRNA amplicon sequencing on an Illumina MiSeq platform. QIIME2 (ver. 2018.2) was used for analysis.



For full table, see supplemental CD-ROM (./Manuscript_III/Table_S1.xlsx)

Table S2: Complete list of *bamA* nucleotide sequences obtained from culture pMB18 cultivated with *p*-methylbenzoate or benzoate as well as *bamA* reference sequences of bacterial isolates and environmental samples.



For full table, see supplemental CD-ROM (./Manuscript_III/Table_S2.xlsx)

Qiime_Scripts.docx

Qiime scripts used for processing of raw Illumina MiSeq dara are available from the supplemental CD-ROM (./Manuscript_III/Qiime_Scripts.docx)

Unpublished results

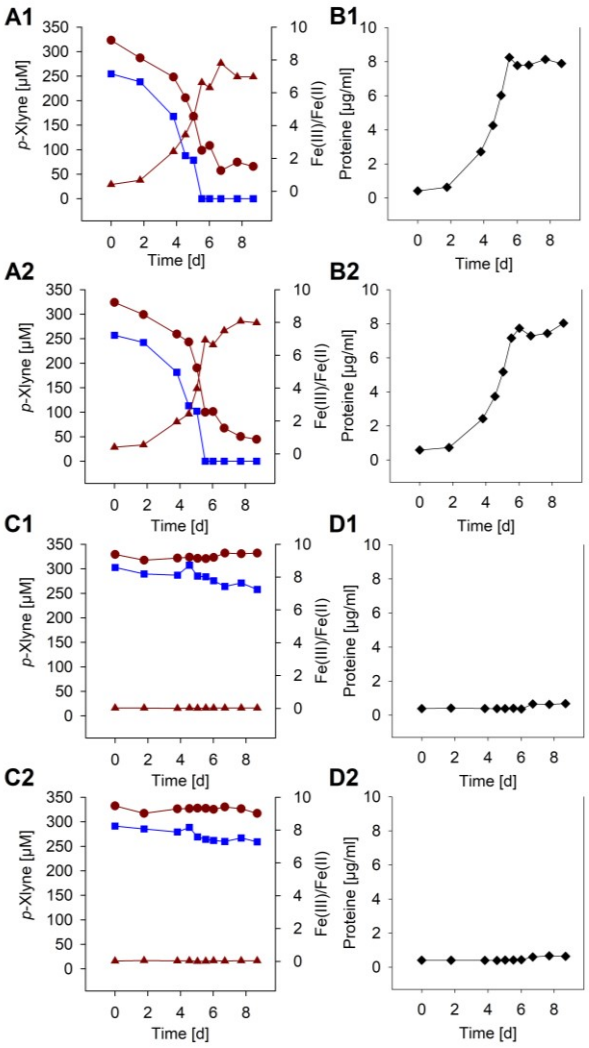


Figure S1: Utilization of *p*-xylene with Fe(III)NTA. The culture was initially enriched with *p*-xylene/nitrate and was transferred for 18 passages with *p*-xylene/Fe(III). 16S rRNA amplicon sequencing (Sanger method) confirmed the predominance of *Georgfuchsia* sp. **(A1-2)** Growth experiments were conducted as duplicates. **(B1-2)** Substrate utilization was coupled to growth. **(C1-2)** No cells were added to negative controls. **(D1-2)** No growth was observed in negative controls. The reduction of Fe(III) to Fe(II) was analyzed as described by Viollier *et al*., 2000. (■) *p*-xylene; (●) Fe(III); (▲) Fe(II); (◆) protein.

Viollier E, Inglett PW, Hunter K., Roychoudhury AN, van Cappellen P (2000) The ferrozine method revisited: Fe(II)/Fe(III) determination in natural waters. *Appl Geochem* 15:785-790.

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VII DECLARATION OF HONOUR

I herewith declare that I am familiar with the relevant course of examination for doctoral candidates. I have composed and written the dissertation by myself and have not used any sections of text from a third party or from dissertations of my own without identifying them as such. Further, I have acknowledged all assistance, personal communication and sources within the work as well as persons that have assisted me with the choice and assessment of materials and/or supported me in writing the manuscript. I have not enlisted the assistance of a doctoral consultant and no third parties have received either direct or indirect monetary benefits from me for work connected to the submitted dissertation. I have not submitted the dissertation as an examination paper for a state or other scientific examination. I have not submitted the same, a substantially similar or any different paper to another postsecondary school.

Martin Sperfeld Jena, 24 May 2018